

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		ATTORNEY'S DOCKET NUMBER 37-02
		U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR not assigned 10/089364
INTERNATIONAL APPLICATION NO PCT/AU00/01183	INTERNATIONAL FILING DATE 22 September 2000	PRIORITY DATE CLAIMED 24 September 1999
TITLE OF INVENTION METHOD OF MODIFYING PLANT PRODUCTIVITY		
APPLICANT(S) FOR DO/EO/US RICHARDSON, Alan Edward; HAYES, Julie Ellen; and SIMPSON, Richard Joseph		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
<ol style="list-style-type: none">1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.3. <input type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.4. <input checked="" type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31).5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371 (c) (2))<ol style="list-style-type: none">a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).b. <input checked="" type="checkbox"/> has been communicated by the International Bureau.c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).6. <input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).<ol style="list-style-type: none">a. <input type="checkbox"/> is attached hereto.b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4).7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))<ol style="list-style-type: none">a. <input checked="" type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).b. <input type="checkbox"/> have been communicated by the International Bureau.c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.d. <input type="checkbox"/> have not been made and will not be made.8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).10. <input type="checkbox"/> An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).11. <input checked="" type="checkbox"/> A copy of the International Preliminary Examination Report (PCT/IPEA/409).12. <input checked="" type="checkbox"/> A copy of the International Search Report (PCT/ISA/210). <p>Items 13 to 20 below concern document(s) or information included:</p> <ol style="list-style-type: none">13. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.14. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.15. <input checked="" type="checkbox"/> A FIRST preliminary amendment.16. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment17. <input type="checkbox"/> A substitute specification.18. <input type="checkbox"/> A change of power of attorney and/or address letter.19. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.20. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4).21. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).22. <input checked="" type="checkbox"/> Certificate of Mailing by Express Mail23. <input type="checkbox"/> Other items or information:		

Page 2 of 2

10/089364-01
PCT Rec'd

09 JUL 2002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Richardson et al.

: Group Art Unit: not assigned

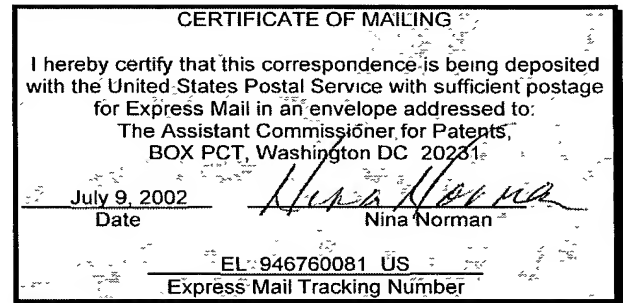
Serial No.: 10/089,364

: Examiner: not assigned

Filed: March 25, 2002

: Confirmation No.: 7785

For: EXPRESSION OF PHYTASE IN
PLANTS AS A METHOD OF
MODIFYING PLANT
PRODUCTIVITY



SECOND PRELIMINARY AMENDMENT

Asst. Commissioner for Patents
Box PCT
Washington, D.C. 20231

Sir:

This amendment accompanies a response to the Notification of Missing Requirements Under 35 U.S.C. 371 in the United States Designated Office (DO/EO/US).

Please amend the application as follows:

In the Specification:

Title

Please correct the title of the invention as: EXPRESSION OF PHYTASE IN PLANTS AS A METHOD OF MODIFYING PLANT PRODUCTIVITY.

REMARKS

The title has been amended to correct an inadvertent error. The amendment made herein does not constitute the addition of new matter.

CONCLUSION

It is believed that no fee is due with the submission of this Second Preliminary Amendment. However, if this is incorrect, please charge the required fee to Deposit Account No. 07-1969.

Respectfully submitted,



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Reg. No. 45,495

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Attorney docket No. 37-02

nrn: July 9, 2002

10/089,364

U.S. Serial No.: 10/089,364

Specification Amendment(s) - Version with markings to show changes made.

TITLE

Please change the title from "Method of Modifying Plant Productivity" to "Expression Of Phytase In Plants As A Method Of Modifying Plant Productivity".

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Richardson et al. : Group Art Unit: not assigned
Serial No.: 10/089,364 : Examiner: not assigned
Filed: March 25, 2002 : Confirmation No.: 7785
For: EXPRESSION OF PHYTASE IN PLANTS AS A METHOD OF MODIFYING
PLANT PRODUCTIVITY

SEQUENCE LISTING TRANSMITTAL
RESPONSE TO NOTIFICATION OF
MISSING REQUIREMENTS UNDER 35 U.S.C. 371

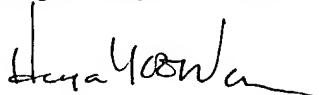
Commissioner for Patents
Box PCT
United States Patent and Trademark Office
Washington, DC 20231

Sir:

The above-identified patent application contains sequences as defined in 37 C.F.R. §1.821(a). Accordingly, the specification includes a paper copy of Sequence Listing as pages 1 - 19. Applicants also submit a write-protected diskette copy of the Sequence Listing in computer-readable form as required by 37 C.F.R. §1.821(e).

In compliance with 37 C.F.R. §1.821(f), the undersigned states that the content of the paper copies and computer-readable copies of the Sequence Listing are the same.

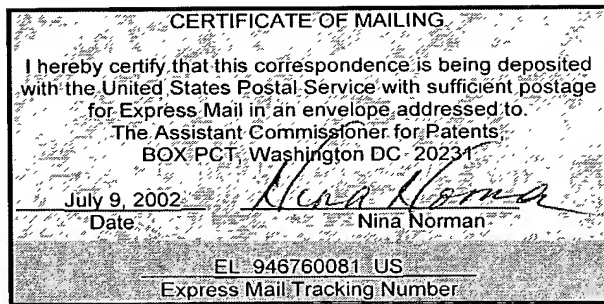
Respectfully submitted,



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Attorney docket No 37-02
nn: July 9, 2002



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Richardson et al.

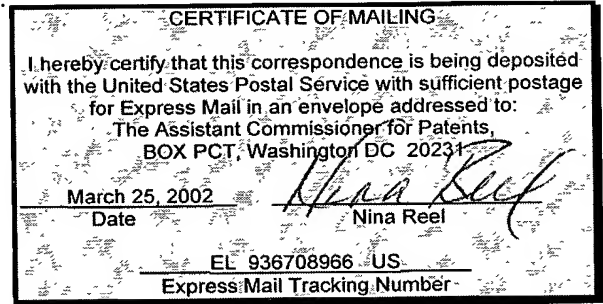
: Group Art Unit: not assigned

Serial No.: not assigned

: Examiner: not assigned

Filed: March 25, 2002

For: METHOD OF MODIFYING PLANT
PRODUCTIVITY



PRELIMINARY AMENDMENT

Asst. Commissioner for Patents
Box PCT
Washington, D.C. 20231

Sir:

This amendment accompanies a filing of a U.S. National Stage application under 35 U.S.C. 371. Filing fees for the National Stage application have been calculated based on this amendment.

Please amend the application as follows:

In the Specification:

Please insert the following on page 1 under the title of the invention.

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a National Stage Application of PCT International Application No. PCT/AU00/01183, filed September 22, 2000, which claims priority from Australian

[illegible]

In the Claims:

Please amend the claims as follows:

5. (Once amended) The method according to any one of claims 1 to 3 wherein the phytase polypeptide has at least about 93% identity to SEQ ID NO: 2.
15. (Once amended) The method according to any one of claims 11, 12 and 14 wherein the phytase polypeptide is from *Aspergillus niger*.
16. (Once amended) The method according to claim 11 or 12 wherein the phytase polypeptide has at least about 93% identity to SEQ ID NO: 2.
23. (Once amended) The method according to any one of claims 11,12, 20, and 21 when used to enhance the phosphorus nutrition of a plant or the growth of a plant on a phosphorus source comprising phytate and/or increase the phosphorus content of a plant.
24. (Once amended) The method according to any one of claims 11,12, 20, and 21 when used to enhance the biomass produced by a plant.
25. (Once amended) The method according to any one of claims 11,12, 20, and 21 when used to enhance the rate of hypocotyl production or the rate of epicotyl production.
39. (Once amended) The process according to claim 34 or 35 wherein the phytase polypeptide is from *Aspergillus niger*.
40. (Once amended) The process according to claim 34 or 35 wherein the phytase polypeptide has at least about 93% identity to SEQ ID NO: 2.

47. (Once amended) The gene construct of claim 46 comprises the *PhyA*-2 gene sequence set forth in SEQ ID NO:1.

In the Sequence Listing:

Please replace the information under the numerical heading of <110> with the following:

Richardson, Alan E., Hayes, Julie E., and Simpson, Richard J.

Please replace the information under the numerical heading of <140> with the following.

PCT/AU00/01183

REMARKS

This application has been amended to add the cross-reference to related applications.

The claims of the International application have been amended to correct formalities. Multiply dependent claims that were dependent upon another multiple dependent claims have been rewritten. This amendment is not intended to change the scope of the claims.

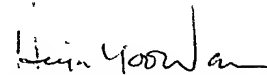
The Sequence Listing has been amended to add the names of the applicants and the serial number of the PCT application to be consistent with the practice of the US patent law.

None of the amendments made herein constitutes the addition of new matter.

CONCLUSION

It is believed that no fee is due with the submission of this Preliminary Amendment. However, if this is incorrect, please charge the required fee to Deposit Account No. 07-1969.

Respectfully submitted,



Heeja Yoo-Warren, Ph.D.
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E-mail: winner@greenwin.com

Attorney docket No. 37-02
nnr: March 25, 2002

U.S. Serial No.: not assigned
Amended Specification - Marked-up version showing changes made.

In the Specification:

Please insert the following on page 1 under the title of the invention.

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a National Stage Application of PCT International Application No. PCT/AU00/01183, filed September 22, 2000, which claims priority from Australian Patent Application No. PQ3049, filed September 24, 1999, both of which are incorporated herein, by reference, in their entirety.

U.S. Serial No.: not assigned

Amended Sequence Listing - Marked-up version showing changes made.

In the Sequence Listing:

Please replace the information under the numerical heading of <110> with the following:

[Commonwealth Scientific and Industrial Research organization and Australian Wool Research and Promotion Organization] Richardson, Alan E., Hayes, Julie E., and Simpson, Richard J.

Please replace the information under the numerical heading of <140> with the following.

[PCT/AU00/XXXXX] PCT/AU00/01183

METHOD OF MODIFYING PLANT PRODUCTIVITY**FIELD OF THE INVENTION**

The present invention relates generally to a method of modifying plant productivity comprising expressing in a plant cell, tissue or organ one or more genes capable of facilitating a plant's ability to utilise soil phosphorus. More particularly, the present invention provides a method of increasing plant productivity comprising expressing in the root of a plant an isolated nucleic acid molecule encoding a phytase polypeptide for a time and under conditions sufficient for said phytase to be secreted from the root, and preferably, further comprising modifying the chemistry of the soil around the root using an organic acid. The present invention extends to novel phytase-encoding genes; genetic constructs which are useful for performing the inventive method; and to transgenic plants produced therewith having improved productivity compared to their otherwise isogenic counterparts.

GENERAL

Those skilled in the art will be aware that the invention described herein is subject to variations and modifications other than those specifically described. It is to be understood that the invention described herein includes all such variations and modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Throughout this specification, unless the context requires otherwise the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps. The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purposes of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

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Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

Reference herein to prior art, including any one or more prior art documents, is not to be taken as an acknowledgment, or suggestion, that said prior art is common general knowledge in Australia or forms a part of the common general knowledge in Australia.

As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

This specification contains nucleotide and amino acid sequence information prepared using the programme PatentIn Version 2.0, presented herein after the claims. Each nucleotide or amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, etc). The length, type of sequence (DNA, protein (PRT), etc) and source organism for each nucleotide or amino acid sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide and amino acid sequences referred to in the specification are defined by descriptor "SEQ ID NO:" followed by the numeric identifier. For example, SEQ ID NO: 1 refers to the information provided in the numeric indicator field designated <400> 1, etc.

The designation of nucleotide residues referred to herein are those recommended by the IUPAC-IUB Biochemical Nomenclature Commission, wherein A represents Adenine, C represents Cytosine, G represents Guanine, T represents thymine, Y represents a pyrimidine residue, R represents a purine residue, M represents Adenine or Cytosine, K represents Guanine or Thymine, S represents Guanine or Cytosine, W represents Adenine or Thymine, H represents a nucleotide other than Guanine, B represents a nucleotide other than Adenine, V represents a nucleotide

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other than Thymine, D represents a nucleotide other than Cytosine and N represents any nucleotide residue.

The designation of amino acid residues referred to herein are also those recommended by the IUPAC-IUB Biochemical Nomenclature Commission, wherein three-letter and one-letter abbreviations for naturally-occurring amino acids are listed in Table 1. In addition to the abbreviations listed in Table 1, the three-letter symbol Asx, or the one-letter symbol B, denotes Asp or Asn; and the three-letter symbol Glx, or the one-letter symbol Z, denotes glutamic acid or glutamine or a substance, such as, for example, 4-carboxyglutamic acid (Gla) or 5-oxoproline (Glp) that yields glutamic acid upon the acid hydrolysis of a peptide.

The designation of plasmid pBS389 herein shall be taken to mean the plasmid depicted in Figure 3, which is also known by those skilled in the art as plasmid pPLEX502, and includes the SCSV promoter and terminator sequences taught in International Patent Application No. PCT/AU95/00552.

Amino acid designations referred to herein are listed in Table 1.

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TABLE 1

	Amino Acid	Three-letter Abbreviation	One-letter Symbol
5	Alanine	Ala	A
	Arginine	Arg	R
	Asparagine	Asn	N
	Aspartic acid	Asp	D
10	Cysteine	Cys	C
	Glutamine	Gln	Q
	Glutamic acid	Glu	E
	Glycine	Gly	G
	Histidine	His	H
15	Isoleucine	Ile	I
	Leucine	Leu	L
	Lysine	Lys	K
	Methionine	Met	M
	Phenylalanine	Phe	F
20	Proline	Pro	P
	Serine	Ser	S
	Threonine	Thr	T
	Tryptophan	Trp	W
	Tyrosine	Tyr	Y
25	Valine	Val	V
	Any amino acid as above	Xaa	X

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BACKGROUND TO THE INVENTION

In light of the dwindling supply of land available for agriculture, intensive agriculture production is an imperative for the purposes of feeding the increasing worldwide population. To achieve this end, it is necessary to increase the productivity of agriculture plants. High productivity is of great agricultural and horticultural value, because increased growth reduce times-to-harvest and yield of crop plants. This improvement is of considerable value in the case of both forage and grain crops.

It is well known that phosphorus may boost or even optimise plant productivity. Soil phosphorus may originate from the deposition of organic material in the soil, which form can account for at least 50-85% of total soil phosphorus. However, organic forms of soil phosphorus, such as, for example, inositol phosphorus (soil phytate), may also account for a substantial component of total soil phosphorus. For example, in Australian soils, phytate accounts for up to 38% of total organic phosphorus, and organic phosphorus may account for 50-85% of total soil phosphorus.

Present methods for boosting plant productivity include the application of phosphate-based fertilisers to the soil. High costs of intensive agriculture, particularly in respect of producing agronomically-important crops, are incurred by the requirement to apply phosphate-based fertilisers to the soil. This is especially evident in regions where the soils are deficient in forms of phosphorus that are readily utilisable by plants. Additionally, there is a considerable environmental cost associated with the use of phosphate fertilisers in particular, due to run-off entering the water catchment and resulting in algal blooms under appropriate conditions.

In spite of the benefits to be derived from providing phosphorus to plants in terms of increased productivity, the use of phosphate-based fertilisers has declined recently, in part due to the high economic costs associated therewith and in part due to the high environmental costs. This decline in phosphate-based fertiliser usage has occurred in regions where soils are deficient in forms of phosphate that are

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available to plants. This has meant a reduction in plant productivity, particularly in those regions having phosphorus-deficient soils.

Notwithstanding the high proportion of total phosphorus present in the soil in the form of soil phytate, plants almost exclusively derive their phosphorus requirement from soluble phosphate anions, and possess a very limited capacity to directly obtain phosphorus from soil phytate, because phytate is not absorbed by plant roots and further, because phytate is inefficiently hydrolysed to inositol and phosphorus in the soil.

10

STATEMENT OF THE PRIOR ART

Microorganisms and fungi in the soil are known to contain phytase enzymes that catalyse the conversion of phytate to inositol and inorganic phosphate and phytase-encoding genes of *Aspergillus niger* have been described previously in United States Patent No. 5, 436, 156 issued on 25 July, 1995 (hereinafter "Van Gorcom *et al.*, 1995").

Additionally, Van Ooijen *et al.* (United States Patent No. 5, 593, 963 issued on 14 January, 1997) expressed the *Aspergillus ssp.* phytase gene in the cells of plants, in particular the seeds, with a view to increasing the level of available phosphorus in feedstock containing the transgenic plants. However, the transgenic plants produced by Van Ooijen *et al.* do not possess improved phosphorus nutrition by virtue of an ability to *utilise* soil phytate.

Hayes *et al* (1999) measured the phytase and acid phosphatase activities in root extracts of burr medic, phalaris, white clover, and subterranean clover. These authors concluded that phytase activity was less than 5% of the total acid phosphatase activity in extracts of roots of these plant species. The authors also characterised the biochemical properties of the phytase enzyme in root extracts of a phosphorus-deficient subterranean clover plant, and showed that the enzyme was inhibited by cobalt, zinc, or arsenate, and suggested that cysteine and EDTA may be effective in the chelation of heavy metals that interfere with phytase activity.

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Hayes *et al* (1999) concluded that a number of pasture plants have limited ability to use phytate-derived phosphorus as a substrate for growth, consistent with the earlier conclusions of Hübel and Beck (1996) that phytase was unlikely to play a role in the phosphorus nutrition of *Zea mays*, notwithstanding that phytase enzyme and phytate were measurable in the root of that species.

SUMMARY OF THE INVENTION

In work leading up to the present invention, the present inventors sought to improve the phosphorus nutrition and yield of plants without the extensive application of phosphate-based fertilisers, by increasing or improving the ability of a plant to utilise phytate, in particular soil phytate. Surprisingly, the present inventors have found that by ectopically expressing phytase enzyme in the roots, and secreting the phytase into the extracellular environment outside the root, the ability of the plant to utilise phytate as a source of phosphorus is markedly improved. Plants produced according to the inventive method provide considerable benefits to the agriculture sector, in the form of reduced economic and environmental costs, and improved plant productivity relative to their otherwise isogenic counterparts. These benefits are further enhanced if the inventive method is coupled with the step of modifying the chemistry of the soil around the root using an organic acid.

Accordingly, one aspect of the invention provides a method of enhancing the phosphorus nutrition of a plant comprising ectopically expressing in the root of a plant an isolated nucleic acid molecule encoding a phytase polypeptide for a time and under conditions sufficient for said phytase to be secreted from the root.

In a preferred embodiment of the invention, the phytase enzyme is secreted from root cells in the region of the root tip and/or the zone of elongation, that divide more rapidly than those cells that are more distal to the root tip.

Preferably, the secretion of phytase from the root provides a high local concentration of active phytase enzyme in the vicinity surrounding those root cells that are involved in active phosphorus uptake. In work leading up to the present

invention the inventors found that mere cell damage or sloughing that occurs during the movement of the root through the soil fails to provide sufficient phytase activity in the region surrounding those root cells involved in phosphorus uptake, and that an active secretion mechanism is important to achieve the improved phosphorus nutrition of the invention. Accordingly, a preferred embodiment of the present invention provides for the phytase enzyme to be produced as a fusion polypeptide with a secretory signal sequence that is active in plant cells and capable of achieving protein transport not merely outside of a root cell, but outside the root surface. In a particularly preferred embodiment, the phytase enzyme is produced as a fusion polypeptide with the leader sequence of the carrot extensin polypeptide to facilitate extracellular targeting of phytase outside the root surface.

In an alternative embodiment, the present invention provides a method of enhancing the phosphorus nutrition of a plant comprising:

- (i) ectopically expressing in the root of a plant an isolated nucleic acid molecule encoding a phytase polypeptide for a time and under conditions sufficient for said phytase to be secreted from the root; and
- (ii) modifying the chemistry of the soil around the root or other growth medium around the root using an organic acid, preferably for a time and under conditions sufficient to solubilise phosphorus produced by the action of said phytase enzyme on phytate, or preferably for a time and under conditions sufficient to make the phytate accessible to the phytase enzyme.

The application of the inventive method results in the production of plants having higher biomass production and/or increased phosphorus content compared to otherwise isogenic counterparts, including higher rates of hypocotyl and epicotyl production, leading to a greater accumulation of biomass and larger plants, without the need for extensive application of phosphate-based fertilisers in soils that comprise phytate or to which phytate has accumulated as a result of past agricultural practice. By virtue of its relative insolubility compared to phosphate anions in soil, phytate-based fertilizers may also provide environmental advantages

- 9 -

relative to super-phosphate based fertilizers. It is also known that phytate is abundant in the excreta of animals, particularly monogastric animals, such as, for example, pigs and poultry, wherein animal excreta represent a significant source of phosphorus contamination into the environment. The inventive method described
5 herein provides a significant solution to the problems of using phytate as a fertilizer for plants, from both the perspective of unlocking those phytate reserves in soils and animal excreta, and from the perspective of reducing environmental contamination associated with the reliance upon superphosphates. Accordingly, the present invention clearly provides for the production and use of "phytate-based
10 fertilisers" in conjunction with the inventive method.

Accordingly, a further aspect of the invention contemplates a plant fertiliser comprising phytate and/or a fertiliser composition comprising phytate and a suitable carrier for application to plants and/or the soil.

15

The present invention further extends to the plants produced by the performance of the inventive method.

A further aspect of the present invention provides an isolated nucleic acid molecule
20 encoding a phytase polypeptide and having more than about 92% nucleotide sequence identity to SEQ ID NO: 1 and/or which is capable of hybridising to SEQ ID NO: 1 or a complementary nucleotide sequence thereto under high stringency hybridisation conditions. Preferably, the isolated nucleic acid molecule of the invention is derived from a microbial source such as, for example, the filamentous
25 fungi *Aspergillus* ssp.

Alternatively, the isolated nucleic acid molecule comprises a nucleotide sequence that encodes an amino acid sequence having more than about 95% identity to the sequence set forth in SEQ ID NO: 2 or an enzymically-active fragment thereof.

30

This aspect of the invention does not extend to the publicly available *PhyA-1* gene of *Aspergillus niger* (GenBank Accession No. M94550; SEQ ID NO: 3),

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notwithstanding that the invention clearly extends to the use of the *PhyA-1* gene or the *PhyA-2* gene in performing the inventive method described herein.

In one embodiment, the isolated nucleic acid molecule encoding phytase is obtainable by the method of:

- a) hybridising under at least low stringency conditions plant genomic DNA, RNA or cDNA derived therefrom with one or more nucleic acid probes or primers of at least 10 nucleotides in length for a period of time and under conditions sufficient to form a double-stranded nucleic acid molecule, wherein said probes or primers comprise a nucleotide sequence obtainable from SEQ ID NO: 1 or a nucleotide sequence that is complementary thereto;
- b) detecting the hybridised nucleic acid molecule; and
- c) isolating said hybridised nucleic acid molecule comprising said genetic sequence.

15

In a particularly preferred embodiment, the isolated nucleic acid molecule of the invention comprises or consists of the nucleotide sequence set forth in SEQ ID NO: 1 or a fragment thereof encoding an active phytase enzyme. Alternatively, the isolated nucleic acid molecule comprises or consists of a nucleotide sequence that encodes the amino acid sequence set forth in SEQ ID NO: 2 or an enzymically-active fragment thereof.

20

A further aspect of the present invention extends to gene constructs comprising a phytase-encoding nucleotide sequence connected in-frame to a secretory signal-encoding nucleotide sequence, and placed operably in connection with a promoter sequence that is operable in the root cells of a plant. Preferably, the phytase-encoding nucleotide sequence comprises or consists of the *Aspergillus niger PhyA-2* nucleotide sequence set forth in SEQ ID NO: 1 or a homologue or derivative thereof as described herein.

30

Preferably, the secretory-signal-encoding nucleotide sequence is the carrot extensin secretory signal or equivalent.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a copy of a representation of a nucleotide sequence alignment between the open reading frames of the chimeric genes produced between the 99 bp nucleotide sequence encoding the carrot extensin leader sequence (bold type) and either the *Aspergillus niger* *PhyA-1* gene (*PhyA-1.seq*; GenBank Accession No. M94550; SEQ ID NO: 3) or the *A. niger* *PhyA-2* gene (*PhyA-2.seq*; SEQ ID NO: 1) obtained by the present inventors. The alignment was produced using the CLUSTAL W algorithm of Thompson *et al* (1994). Numbering refers to the nucleotide positions from the start of the chimeric *ext::PhyA* genes. Bars between the sequences represent identical nucleotide residues.

Figure 2 is a copy of a representation of an amino acid sequence alignment between two fusion polypeptides comprising the carrot extensin leader sequence (bold type) fused to either the *Aspergillus niger* *PhyA-1* polypeptide (*PhyA-1.pro*; GenBank Accession No. M94550; SEQ ID NO: 4) or the *A. niger* *PhyA-2* polypeptide (*PhyA-2.pro*; SEQ ID NO: 2) obtained by the present inventors. The alignment was produced using the CLUSTAL W algorithm of Thompson *et al* (1994). Numbering refers to the amino acid positions from the start of the chimeric polypeptides. Bars between the sequences represent identical amino acid residues.

Figure 3 is a copy of a representation of the pPLEX vector plasmid designated pBS389. This plasmid contains the sub-clover stunt virus (SCSV) region 1 promoter (Sc1 Pr; International Patent Application No. PCT/AU95/00552) and SCSV region 3 terminator (Sc3 3'; International Patent Application No. PCT/AU95/00552) operably connected to a kanamycin-resistance gene (*nptII*) for expression in plants, flanked by the *Agrobacterium tumefaciens* left-border (LB) and right-border (RB) integration sequences; a bacterial-operable spectinomycin/streptomycin resistance gene (*Sp-R/St-R*); an *Agrobacterium* origin of replication (*oriVRK2*) and *E.coli* origin of

replication (oriColE1) and intergenic spacer (IS1/oriT). Positions of restriction sites are indicated.

Figure 4 is a copy of a representation of the plasmid pART 7. Plasmid pART7 is a vector containing bacterial two origins of replication (f1 ori and ori), an ampicillin resistance gene for bacterial selection (Amp^R). Plasmid pART7 also contains two *NotI* restriction sites flanking a CaMV 35S promoter-multiple cloning site(MCS)-NOS 3' cassette, wherein the MCS permits cloning of structural genes in operable connection with said promoter and terminator sequences.

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Figure 5 is a copy of a representation of the plasmid pAER02, containing the carrot extensin leader-encoding sequence (ext) in-frame with the *A. niger PhyA-1* gene (PhyA-1; SEQ ID NO: 3) and placed operably in connection with the CaMV 35S promoter sequence and OCS terminator sequence. This vector is based upon plasmid pBS389 (Figure 3).

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Figure 6 is a copy of a representation of the plasmid pAER04, containing the carrot extensin leader-encoding sequence (ext) in-frame with the *A. niger PhyA-2* gene (PhyA-2; SEQ ID NO: 1) and placed operably in connection with the CaMV 35S promoter sequence and OCS terminator sequence. This vector is based upon plasmid pBS389 (Figure 3).

20

Figure 7 is a photographic representation showing the growth of transgenic *Arabidopsis thaliana* (C24 ecotype) plants (T1 generation) containing a binary vector selected from the group consisting of: (i) plasmid pBS389 (row marked "A"); (ii) a plasmid containing the *PhyA-2* gene (SEQ ID NO: 1) in plasmid pBS389 (no leader sequence; row marked "B"); and (iii) plasmid pAER04 (Figure 6; row marked "C"). Transgenic plants were grown for 42 days, at a density of three plants per tube, in sterile agar media supplemented with 0.8 mM Na₂HPO₄ (column marked "Phosphate"), or phytate, equivalent to 0.8 mM phosphate (column marked "Phytate"), or without added phosphorus (column marked "No P"). Duplicate results are indicated for each condition stated herein.

25

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Figure 8 is a photographic representation showing growth on phytate-containing agar media, of transgenic *A. thaliana* (C24 ecotype) plants containing a binary vector selected from the group consisting of: (A) plasmid pBS389 ; (B) a plasmid containing the *PhyA-1* gene (SEQ ID NO: 3) in plasmid pBS389 (no leader sequence); (C) plasmid pAER02 (Figure 5); (D) a plasmid containing the *PhyA-2* gene (SEQ ID NO: 1) in plasmid pBS389 (no leader sequence); and (E) plasmid pAER04 (Figure 6). Transgenic plants were grown for 42 days, at a density of three plants per tube, in sterile agar media supplemented with phytate, equivalent to 0.8 mM phosphate. Duplicate results are indicated for each condition stated herein.

Figure 9 is a photographic representation showing growth on media lacking added phosphorus (panel A), or on phytate-containing agar media having a phytate concentration equivalent to 0.8 mM phosphate (panels B, C, D), of transgenic *A. thaliana* (C24 ecotype) plants containing a binary vector selected from the group consisting of: (A) plasmid pAER02 (Figure 5); (B) a plasmid containing the *PhyA-1* gene (SEQ ID NO: 3) in plasmid pBS389 (no leader sequence); (C) plasmid pAER04 (Figure 6); and (D) plasmid pAER02 (Figure 5). Plants were grown for 40 days at a density of about thirty plants per agar plate.

Figure 10 is a photographic representation showing growth on sterile agar media plates supplemented with phytate at a concentration equivalent to 0.8 mM phosphate (panels A, C), or 0.8 mM Na_2HPO_4 (panel B), of transgenic *A. thaliana* (C24 ecotype) plants containing a binary vector selected from the group consisting of: (A) a plasmid containing the *PhyA-1* gene (SEQ ID NO: 3) in plasmid pBS389 (no leader sequence); (B) plasmid pAER02 (Figure 5); and (C) plasmid pAER02 (Figure 5). Plants were grown for 22 days and plates were stained with 0.03% (w/v) FeCl_3 to determine regions where phytate was absent from the medium. Regions of darker shading in the plates, in particular the heavier regions along the margins of the roots in Panel C show the absence of phytate from the medium, which phytate has been utilised by the plants.

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Figure 11 is a photographic representation of a northern blot experiment showing ectopic expression of phytase genes in transgenic *A. thaliana* (C24 ecotype) plants. The upper panel is an ethidium bromide-stained agarose gel containing 10 µg of total RNA isolated from transgenic shoots. The lower panel shows an

5 autoradiograph of the same mRNAs following hybridization under stringent conditions [e.g. at 65°C, in a buffer comprising 0.5xSSC and 0.1% (w/v) SDS] with the *PhyA-1* sequence (SEQ ID NO: 3). The mRNA samples indicated were derived from lines of transformed plants containing a plasmid selected from the group consisting of: (A) plasmid pBS389 ; (B) a plasmid containing the *PhyA-2* gene (SEQ

10 ID NO: 1) in plasmid pBS389 (no leader sequence); (C) plasmid pAER04 (Figure 6); (D) a plasmid containing the *PhyA-1* gene (SEQ ID NO: 3) in plasmid pBS389 (no leader sequence); and (E) plasmid pAER02 (Figure 5). Duplicate lines are indicated for each plasmid. For RNA isolation, the plants were grown for 36 days on sterile agar supplied with 0.8 mM phosphorus.

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Figure 12 is a photographic representation of a Southern blot of total DNA from transgenic *A. thaliana* (C24 ecotype) plants produced using a plasmid selected from the group consisting of: (A) plasmid pBS389 ; (B) a plasmid containing the *PhyA-2* gene (SEQ ID NO: 1) in plasmid pBS389 (no leader sequence); (C) plasmid

20 pAER04 (Figure 6); (D) a plasmid containing the *PhyA-1* gene (SEQ ID NO: 3) in plasmid pBS389 (no leader sequence); and (E) plasmid pAER02 (Figure 5). DNA samples (~6 µg digested with *EcoRI*) were hybridized under stringent conditions [e.g. at 65°C, in a buffer comprising 0.5xSSC and 0.5% (w/v) SDS] with the *PhyA-1* sequence (SEQ ID NO: 3). Multiple lines are indicated for C, D, and E. For DNA

25 isolation, the plants were grown for 36 days on sterile agar supplied with 0.8 mM phosphorus. Size markers (kb) are indicated at the left of the Figure. The lane marked # is a control lane containing DNA from non-transgenic *A. thaliana*.

Figure 13 is a photographic representation of a northern blot experiment showing

30 ectopic expression of phytase genes in transgenic *Trifolium subterraneum* (subterranean clover, cultivar Dalkeith) plants (T₀ generation). The upper panel is an ethidium bromide-stained agarose gel containing 10 µg of total RNA isolated

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from transgenic shoots. The centre panel shows an autoradiograph of the same mRNAs following hybridization under stringent conditions [e.g. at 65°C, in a buffer comprising 0.5xSSC and 0.1% (w/v) SDS] with the *PhyA-1* sequence (SEQ ID NO: 3). The lower panel indicates the number of transgene inserts as determined by Southern blot analysis (not shown). The mRNA samples indicated were derived from 10 lines of transformed plants, of which five were independent lines (lines i, ii, iii, iv and v), produced by transformation of explants with the plasmid pAER02 (Figure 5). The lane marked # is a positive control containing RNA from transgenic *A. thaliana* plants produced using the vector pAER02 (see Figure 12E). For RNA isolation, tissue culture-derived explant material was grown to maturity under glasshouse conditions.

Figure 14 is a graphical representation showing the release of phosphorus from soil by extraction of air-dried soil in 50 mM citric acid. **Panel (a):** The hydrolysis of organic phosphorus in soil extracts was measured for up to 8 hr in the presence of either no added phytase enzyme (♦), or alternatively, in the presence of different concentrations of commercially-available phytase enzyme, as follows: (a) 0.005 nkat phytase g⁻¹ soil (Δ); (b) 0.05 nkat phytase g⁻¹ soil (○); or (c) 0.50 nkat phytase g⁻¹ soil (■). **Panel (b):** The hydrolysis of organic phosphorus in soil extracts was measured for up to 8 hr in the presence of either no added phytase enzyme (♦), or alternatively, in the presence of different concentrations of purified phytase enzyme, as follows: (a) 0.23 nkat phytase g⁻¹ soil (□); or (b) 2.28 nkat phytase g⁻¹ soil (■). Soil was from Rutherglen Research Institute, Victoria, Australia, and had been fertilised with superphosphate (9% phosphorus) at a rate of 125 kg ha⁻¹ each alternate year since 1914. Time (hr) is indicated on the abscissa, and phosphorus (μg phosphorus (P) g⁻¹ soil) is indicated on the ordinate. Each point represents the mean of three observations. Bars indicate standard error and, where not shown, were smaller than the symbol.

Figure 15 is a graphical representation showing the effect of citric acid on the hydrolysis of phytate in air-dried soil, as measured by the release of phytase-labile organic phosphorus. The hydrolysis of organic phosphorus in soil extracts was

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measured for 6 hr in the presence of either 0.50 nkat commercial phytase g⁻¹ soil (■), or 2.28 nkat purified phytase g⁻¹ soil (□), and various concentrations of citric acid indicated on the abscissa. Phytase-labile organic phosphorus (µg phosphorus (P) g⁻¹ soil) is indicated on the ordinate. Total extractable organic phosphorus (µg phosphorus g⁻¹ soil) is also shown in the figure (○). Soil was from Rutherglen Research Institute, Victoria, Australia, and had been fertilised with superphosphate (9% phosphorus) at a rate of 125 kg ha⁻¹ each alternate year since 1914. Each point represents the mean of three observations. Bars indicate standard error and, where not shown, were smaller than the symbol. Data show a significant positive correlation between the hydrolysis of phytate and the concentration of citric acid in the soil extract.

Figure 16 is a graphical representation showing the effect of pH on the hydrolysis of phytate in air-dried soil, as measured by the release of phytase-labile organic phosphorus. The hydrolysis of organic phosphorus in soil extracts was measured for 6 hr in the presence of either 0.50 nkat commercial phytase g⁻¹ soil (■), or 2.28 nkat purified phytase g⁻¹ soil (□), in the presence of 50 mM citric acid at the pH values indicated on the abscissa. Phytase-labile organic phosphorus (µg phosphorus (P) g⁻¹ soil) is indicated on the ordinate. Total extractable organic phosphorus (µg phosphorus g⁻¹ soil) is also shown in the figure (○). Soil was from Rutherglen Research Institute, Victoria, Australia, and had been fertilised with superphosphate (9% phosphorus) at a rate of 125 kg ha⁻¹ each alternate year since 1914. Each point represents the mean of three observations. Bars indicate standard error and, where not shown, were smaller than the symbol. Data show that, whilst extractable phosphorus increased with pH, the hydrolysis of phytate by phytase was similar across the range of pH values tested.

Figure 17 is a graphical representation showing the organic phosphorus (filled bars) or inorganic phosphorus (open bars) contents of soils from two sites in Australia that are extractable by water (panels a, b), 50 mM citric acid (panels c, d), or 500 mM sodium bicarbonate (pH 8.5; panels e, f); and the organic phosphorus that was hydrolysed by phytase in soil extracts for 6 hr in the presence of either 0.50 nkat

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commercial phytase g^{-1} soil (open-hatched bars), or 2.28 nkat purified phytase g^{-1} soil (densely-hatched bars), in each of the above conditions. One soil sample was from Rutherglen Research Institute, Victoria, Australia, that had been either unfertilised (U_R), or fertilised with superphosphate (9% phosphorus) at a rate of 125 kg ha^{-1} each alternate year since 1914 (F_R), or fertilised as for F_R soils and also top-dressed with nine applications of lime at the rate of 1.25 tonnes ha^{-1} in the period 1914-1948 ($F+L_R$). The other soil sample was from Ginninderra Experiment Station (Wallaroo 3 paddock) that had been either unfertilised (U_G), or received three autumn applications of triple superphosphate (20.7% phosphorus) at a rate of either 416 kg ha^{-1} ($F1_G$) or 675 kg ha^{-1} ($F2_G$). Extractable phosphorus (μg phosphorus g^{-1} soil) is indicated on the ordinate. Each bar represents the mean of three observations.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

- One aspect of the invention provides a method of improving the phosphorus nutrition of a plant comprising ectopically expressing in the root of a plant an isolated nucleic acid molecule encoding a phytase polypeptide for a time and under conditions sufficient for said phytase to be secreted from the root.
- As used herein, the term "phosphorus nutrition" shall be taken to refer to the utilisation by a plant of an external source of phosphorus in any form, including organic phosphorus and/or phosphate anion and/or phytate and/or phosphate and/or orthophosphate and/or pyrophosphate, amongst others. By "external source of phosphorus" is meant phosphorus that is taken up by the plant from the external environment.

An "improved phosphorus nutrition" refers to a greater ability of the plant to utilise an existing phosphorus source in the soil or growth medium in which said plant grows.

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Accordingly, the present invention is directed to a method of improving the ability of a plant to utilise an external source of phosphorus. In a particularly preferred embodiment, the external source of phosphorus is phytate.

- 5 The term "phytate" shall be taken to refer to any storage phosphorus source comprising inositol phosphate, including aggregates and polymers thereof, and phytin, a generic term applied to complex salts of phytic acid (for a review see Graf, 1986).
- 10 The term "phytase polypeptide" refers to any amino acid sequence, peptide, oligopeptide, polypeptide, or protein molecule, with or without additional non-amino acid substituents or non-naturally-occurring amino acid substituents, that is capable of catalysing the removing a phosphate-containing moiety from phytate as hereinbefore defined. Preferably, a phytase polypeptide will further be capable of
- 15 catalysing the conversion of phytate to inositol and phosphate, which may be in any form, such as, for example, an anion, or metal complex, a transition metal complex, or a weak acid, amongst others. Those skilled in the art will be aware of those forms of soil phosphate that are readily utilisable by plants, and the present invention clearly extends to phytase enzymes capable of converting phytate to any such form
- 20 of soil phosphate.

Whilst the present invention is not limited by the source of phytase, the phytase polypeptide of the invention is preferably derived from a plant, microorganism, or animal cell. Those skilled in the art will be aware that phytases are widely-occurring

25 enzymes in nature, derivable from bacteria, such as, for example, *Bacillus subtilis* (Paver and Jagannathan, 1982), *Pseudomonas* (Cosgrove, 1970); yeasts, such as, for example, *Saccharomyces cerevisiae* (Nayini and Markakis, 1984); fungi, such, for example, *Aspergillus fumigatus* (Wyss *et al.*, 1999), *Aspergillus terreus* (Yamada *et al.*, 1986), *A. niger* (Mullaney *et al.*, 1991; van Hartingsveldt *et al.*, 1993); plants

30 (Loewus, 1990) such as, for example, maize (Laboure *et al.*, 1993; Hubel and Beck, 1996; Maugenest *et al.*, 1997), potato (Gellady and Lefebvre, 1990), and soybean (Gibson *et al.*, 1988), amongst others. Conveniently, the phytase enzyme

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employed in the performance of the present invention possesses high specific activity and/or high Vmax in a soil environment and/or low Km for phytate in a soil environment.

- 5 In a particularly preferred embodiment, the phytase enzyme employed in the performance of the present invention is derived from a fungus, more preferably *Aspergillus spp.*, and even more preferably from *A. niger*. As exemplified herein, the *A. niger* phytase enzymes comprising the amino acid sequences set forth in SEQ ID NO: 2 or SEQ ID NO: 4 provides high biomass production and/or increased
10 phosphorus content when expressed in the roots of transgenic plants and secreted therefrom into the surrounding growth medium.

For the purposes of nomenclature, the amino acid sequence set forth in SEQ ID NO: 2 relates to the *A. niger* PhyA-2 phytase polypeptide, which has been
15 produced by expression of the *A. niger* PhyA-2 gene (SEQ ID NO: 1). The present inventors have modified the naturally-occurring gene to be more suitable for expression in plants.

The amino acid sequence set forth in SEQ ID NO: 4 relates to a variant of the *A. niger* PhyA-1 polypeptide (Mullaney *et al.*, 1991; Van Hartingsveldt *et al.*, 1993; GenBank Accession No. M94550), having the leader sequence removed and a different translation start site inserted relative to the naturally-occurring PhyA-1 polypeptide. To express the PhyA-1 polypeptide in plants, the present inventors modified the corresponding *PhyA-1* gene sequence to remove the endogenous *A. niger* leader sequence-encoding nucleotide sequence and intron sequence, and
25 introduced a new translation start site immediately prior to and in-frame with, the nucleotide sequence encoding the mature PhyA-1 polypeptide.

As used herein, the terms "in-frame" and "in the same reading frame" refer to one or
30 more codons of a nucleotide sequence being in the same open reading frame as one or more other codons of said nucleotide sequence. Similarly, the term "in-frame fusion" refers to the linkage between two or more heterologous nucleotide

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sequences such that the amino acid sequences encoded thereby are expressed in the same reading frame and, as a consequence, as a single polypeptide molecule.

5 The phytase polypeptide may be expressed throughout the length of the root, or alternatively, in a localised region of the root, preferably in the zone of elongation and/or the root tip. Conveniently, expression occurs in the epidermal cells and/or the cortex, to facilitate the secretion of the phytase to the root surface, either by reducing the number of cell layers through which the phytase must be transported or alternatively, by facilitating transport of the phytase to the epidermal cells.

10

Preferably, secretion of the phytase polypeptide from the root cells in which it is expressed is achieved by expressing the phytase as an in-frame fusion polypeptide with a secretory signal sequence capable of directing transport of the phytase to the root surface. According to this embodiment, the secretory signal sequence may be placed at the N-terminal and/or C-terminal end of the phytase polypeptide. Those skilled in the art will be aware that such signal sequences have been demonstrated to be operable in either configuration. Alternatively, or in addition, a secretory signal sequence may be embedded in the phytase polypeptide, the only requirement being that the embedding of the secretory signal sequence in the phytase does not inactivate the phytase enzymic activity in the soil or growth medium. The present invention further encompasses the use of a cryptic secretory signal sequence, either as an in-frame fusion with phytase or alternatively, by mutation of a region of the phytase polypeptide to produce an amino acid variant phytase polypeptide, such as a substitutional variant or insertional variant or deletional variant, that comprises a cryptic secretory signal sequence therein.

25

Substitutional variants are those in which at least one residue in the phytase amino acid sequence has been removed and a different residue inserted in its place. Amino acid substitutions are typically of single residues, but may be clustered depending upon functional constraints placed upon the polypeptide; insertions will usually be of the order of about 1-10 amino acid residues. and deletions will range

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in-frame fusion with the secretory signal sequence derived from the carrot extensin protein, exhibit improved growth on phytate relative to otherwise isogenic non-transformed plants. Using such tests, those skilled in the art can readily determine the optimum secretory signal sequence for performing the present invention, and
5 the optimum placement of said secretory signal sequence relative to the phytase polypeptide.

The secretory signal sequence is conveniently derived from the full-length potato patatin polypeptide (Iturriaga *et al.*, 1989; Li *et al.*, 1997), the tobacco PR-S
10 polypeptide (Comelissen *et al.* 1986; Pen *et al.*, 1993), the lupin acid phosphatase (LASAP 1; Wasaki *et al.*, 1999) polypeptide or the carrot extensin polypeptide (Chen and Varner, 1985).

In a particularly preferred embodiment, the secretory signal sequence is derived
15 from the carrot extensin polypeptide and placed at the N-terminus of the phytase polypeptide.

Nucleotide sequences of the secretory signal-encoding nucleotide sequences of the carrot extensin and lupin acid phosphatase genes are set forth herein, as SEQ ID
20 NOs: 5 and 7, respectively. The amino acid sequences of the carrot extensin and lupin acid phosphatase secretory signal peptides are also set forth herein, as SEQ ID NOs: 6 and 8, respectively.

Additionally, the nucleotide sequences of chimeric genes encoding the carrot
25 extensin leader sequence fused to the *PhyA-2* or *PhyA-1* gene, are shown in Figure 1 and SEQ ID Nos: 9 and 11, respectively. The amino acid sequences of fusion polypeptides comprising the carrot extensin leader sequence fused to the modified *PhyA-2* or *PhyA-1* polypeptides, are shown in Figure 2 and SEQ ID Nos: 10 and 12, respectively. These chimeric genes have been expressed by the inventors in several
30 plant species by the methods described herein to enhance the phosphorus nutrition of those plants.

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The word "express" or variations such as "expressing" and "expression" as used herein shall be taken in their broadest context to refer to the transcription of a particular genetic sequence to produce sense or antisense mRNA or the translation of a sense mRNA molecule to produce a peptide, polypeptide, oligopeptide, protein or enzyme molecule. In the case of expression comprising the production of a sense mRNA transcript, the word "express" or variations such as "expressing" and "expression" may also be construed to indicate the combination of transcription and translation processes, with or without subsequent post-translational events which modify the biological activity, cellular or sub-cellular localisation, turnover or steady-state level of the peptide, polypeptide, oligopeptide, protein or enzyme molecule.

Without being bound by any theory or mode of action, the expression of phytase in the root cell and its subsequent secretion to the root surface provides a localised high concentration of active phytase enzyme the is capable of diffusing into the soil to catalyse the conversion of phytate into inositol and phosphate, such that the phosphate is then able to be absorbed or actively taken up by the root.

The term "ectopic expression" refers to the *de novo* and/or increased expression of a peptide, oligopeptide, polypeptide or protein from an introduced nucleic acid molecule, such as, for example, by means of transfection or transformation of a cell, tissue or organ with nucleic acid encoding the peptide, oligopeptide, polypeptide or protein. Ectopic expression can also be achieved by the infection or transformation of a cell, tissue or organ with a foreign organism containing nucleic acid encoding the peptide, oligopeptide, polypeptide or protein.

Accordingly, to ectopically-express a phytase polypeptide comprising an in-frame fusion with a secretory signal sequence, it is necessary to produce a corresponding nucleic acid molecule encoding both the secretory signal sequence and the phytase polypeptide in the same reading frame. This may be achieved by those skilled in the art without undue experimentation.

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For the ectopic expression of a peptide, oligopeptide, polypeptide or protein in a plant cell, tissue or organ, the nucleic acid molecule encoding said peptide, oligopeptide, polypeptide or protein in a plant-expressible format, such as, for example, in an appropriate gene construct comprising a promoter sequence
5 operably connected to said nucleic acid molecule, and optionally a transcription termination sequence comprising a polyadenylation signal, amongst others.

By "expressible format" is meant that the isolated nucleic acid molecule is in a form suitable for being transcribed into mRNA and/or translated to produce a protein,
10 either constitutively or following induction by an intracellular or extracellular signal, such as an environmental stimulus or stress (anoxia, hypoxia, temperature, salt, light, dehydration, low phosphate, low P, etc) or a chemical compound such as an antibiotic (tetracycline, ampicillin, rifampicin, kanamycin) hormone (eg. gibberellin, auxin, cytokinin, glucocorticoid, etc), hormone analogue (iodoacetic acid (IAA), 2,4-
15 D, etc) , metal (zinc, copper, iron, etc), or dexamethasone, amongst others. As will be known to those skilled in the art, expression of a functional protein may also require one or more post-translational modifications, such as glycosylation, phosphorylation, dephosphorylation, or one or more protein-protein interactions, amongst others. All such processes are included within the scope of the term
20 "expressible format".

The term "plant-expressible format" refers to an expressible format that pertains to expression of proteins in plant cells, tissues or organs.

25 Preferably, the ectopic expression of phytase is effected by introducing an isolated nucleic acid molecule encoding phytase, such as a cDNA molecule, genomic gene, synthetic oligonucleotide molecule, mRNA molecule or open reading frame, to a plant cell, tissue or organ, operably in connection with a promoter sequence that is capable of conferring expression in a plant root cell, albeit not necessarily
30 exclusively in the root cell.

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Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences derived from a classical eukaryotic genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or are capable of conferring expression on a structural gene sequence (i.e. the protein-coding region of a gene) in a tissue-specific manner, conveniently in the roots.

10 In the present context, the term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of a nucleic acid molecule in a plant cell, tissue or organ.

Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression and/or to alter the spatial expression and/or temporal expression of a nucleic acid molecule to which it is operably connected. For example, copper-responsive, glucocorticoid-responsive or dexamethasone-responsive regulatory elements may be placed adjacent to a heterologous promoter sequence driving expression of a nucleic acid molecule to confer copper inducible, glucocorticoid-inducible, or dexamethasone-inducible expression respectively, on said nucleic acid molecule.

Included within the scope of the present invention is the use of strong constitutive promoter sequences, cell-specific promoter sequences, inducible promoter sequence, tissue-specific promoter sequences, organ-specific promoter sequences, and constitutive promoter sequences that have been modified to confer expression in a particular part of the plant at any one time, such as by integration of said constitutive promoter within a transposable genetic element (*Ac*, *Ds*, *Spm*, *En*, or other transposon).

30

The term "constitutive" will be known by those skilled in the art to indicate that expression is observed predominantly throughout the plant, albeit not necessarily in

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every cell, tissue or organ under all conditions. In the present context, a preferred strong constitutive promoter is one which confers a high level of ectopic expression on a phytase structural gene to which it is operably connected, predominantly throughout the plant and at least in the root, albeit not necessarily in every cell, tissue or organ under all conditions.

The term "cell-specific" shall be taken to indicate that expression is predominantly in a particular plant cell or plant cell-type, albeit not necessarily exclusively in that plant cell or plant cell-type. In the present context, a preferred cell-specific promoter will confer expression on a phytase gene in at least one cell type of the root, preferably, a root epidermal cell or root cortical cell.

Similarly, the term "tissue-specific" shall be taken to indicate that expression is predominantly in a particular plant tissue or plant tissue-type, albeit not necessarily exclusively in that plant tissue or plant tissue-type. In the present context, a preferred tissue-specific promoter will confer expression on one or more tissues of the root, such as, for example, in the zone of elongation, the root vasculature, or the root tip.

Similarly, the term "organ-specific" shall be taken to indicate that expression is predominantly in a particular plant organ albeit not necessarily exclusively in that plant organ. In the present context, a preferred organ-specific promoter will confer expression on a phytase gene throughout the root.

Those skilled in the art will be aware that an "inducible promoter" is a promoter the transcriptional activity of which is increased or induced in response to a developmental, chemical or physical stimulus.

Preferably, the promoter is a root-specific, phosphate-regulated promoter derived from a phosphate transport gene, such as, for example, the phosphate transport genes of *A. thaliana* or barley plants, which are induced under conditions of

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phosphate deficiency in the plant. These promoters are obtainable from CSIRO Tropical Agriculture, Queensland, Australia.

5 The present invention is not to be limited by the choice of promoter sequence and those skilled in the art will readily be capable of selecting appropriate promoter sequences for use in regulating appropriate expression of phytase or modified phytase from publicly-available or readily-available sources, without undue experimentation.

10 Placing a phytase-encoding nucleic acid molecule under the regulatory control of a promoter sequence, or in operable connection with a promoter sequence, means positioning said nucleic acid molecule such that expression is controlled by the promoter sequence.

15 A promoter is usually, but not necessarily, positioned upstream, or at the 5'-end, and within 2 kb of the start site of transcription, of the nucleic acid molecule which it regulates.

20 In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting (i.e., the gene from which the promoter is derived). As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred
25 positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting (i.e., the gene from which it is derived). Again, as is known in the art, some variation in this distance can also occur.

30 Examples of strong constitutive promoters and root-specific promoters that are suitable for use in expressing phytase in the roots of plants include those listed in Table 2, amongst others. The promoters listed in Table 2 are provided for the

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purposes of exemplification only and the present invention is not to be limited by the list provided therein. Those skilled in the art will readily be in a position to provide additional promoters that are useful in performing the present invention.

- 5 Preferred tissue-specific inducible promoter sequences include the anoxia-inducible and hypoxia-inducible maize *Adh1* gene promoter (Howard *et al.*, 1987; Walker *et al.*, 1987). Such environmentally-inducible promoters are reviewed in detail by Kuhlemeier *et al.* (1987).
- 10 Preferred chemically-inducible promoters include the 3- β - indoylacrylic acid-inducible *Tip* promoter; IPTG-inducible *lac* promoter; phosphate-inducible promoter; L-arabinose-inducible *araB* promoter; heavy metal-inducible metallothioneine gene promoter; dexamethasone-inducible promoter; glucocorticoid-inducible promoter; ethanol-inducible promoter (Zeneca); the N,N-diallyl-2,2-dichloroacetamide-
- 15 inducible glutathione-S-transferase gene promoter (Wiegand *et al.*, 1986); or any one or more of the chemically-inducible promoters described by Gatz *et al.* (1996;1998), amongst others.

- Preferred wound-inducible or pathogen-inducible promoters include the
- 20 phenylalanine ammonia lyase (PAL) gene promoter (Ebel *et al.*, 1984), chalcone synthase gene promoter (Ebel *et al.*, 1984) or the potato wound-inducible promoter (Cleveland *et al.*, 1987), amongst others.

- In the case of constitutive promoters or promoters that induce expression
- 25 throughout the entire plant, such sequences may be modified by the addition of nucleotide sequences derived from one or more of the root-specific promoters listed in Table 2, and optionally, additional nucleotide sequences derived from one or more inducible promoters, to confer inducible tissue-specificity thereon. For example, the CaMV 35S promoter may be modified by the addition of maize *Adh1*
- 30 promoter sequence, to confer anaerobically-regulated root-specific expression thereon, as described previously (Ellis *et al.*, 1987). Such modifications can be achieved by routine experimentation by those skilled in the art.

In a particularly preferred embodiment of the present invention, the phytase is ectopically expressed under control of the CaMV 35S promoter sequence.

5 In each of the preceding embodiments of the present invention, the phytase protein or a homologue, analogue, or derivative thereof, in particular the *A. niger* phytase protein PhyA-1 or PhyA-2, is expressed under the operable control of a promoter sequence operable in the root. As will be known those skilled in the art, this is generally achieved by introducing a gene construct or vector into plant cells by
10 transformation or transfection means. The nucleic acid molecule or a gene construct comprising same may be introduced into a cell using any known method for the transfection or transformation of said cell. Wherein a cell is transformed by the gene construct of the invention, a whole organism may be regenerated from a single transformed cell, using methods known to those skilled in the art.

15 By "transfect" is meant that the gene construct or vector or an active fragment thereof comprising the *PhyA-1* or *PhyA-2* gene or a homologue, analogue or derivative thereof, operably under the control of the promoter sequence is introduced into said cell without integration into the cell's genome.

20 By "transform" is meant that the gene construct or vector or an active fragment thereof comprising the *PhyA-1* or *PhyA-2* gene or a homologue, analogue or derivative thereof, operably under the control of the plant-expressible promoter sequence is stably integrated into the genome of the cell.

25 In an alternative embodiment, the present invention provides a method of improving the phosphorus nutrition of a plant comprising:

- 30 (i) ectopically expressing in the root of a plant an isolated nucleic acid molecule encoding a phytase polypeptide for a time and under conditions sufficient for said phytase to be secreted from the root; and
- (ii) modifying the chemistry of the soil around the root or other growth medium around the root.

- 30 -

As used herein, the term "modifying the chemistry of the soil around the root or other growth medium around the root using an organic acid" or similar shall be taken to include any effect of an organic acid on increasing the ability of a plant to
5 utilise phytase-labile phosphorus and/or total organic phosphorus, such as, for example, acidification, and/or a chelating effect that facilitates the solubilisation of phosphorus and/or phosphorus uptake, or to make the phytate accessible to the phytase enzyme, amongst others. The present invention is not to be limited by the mode of action of the organic acid in improving phosphorus nutrition, the only
10 requirement being that the amount of organic acid in the vicinity of the root is increased, such as by direct application, extracellular secretion or active transport, amongst others.

The present invention particularly extends to the use of any agent known to those
15 skilled in the art to modify the chemistry of the soil by chelation, preferably by chelation of a metal, such as, for example a transition metal, to facilitate phosphate uptake by a plant. In this regard, phosphate in the soil which is released by the breakdown of phytate may form complexes with various metals in the soil, such as, for example, aluminium. Without being bound by any theory or mode of action, the
20 presence of an organic acid in the vicinity of the root where phytase acts in accordance with the inventive method may increase access of phytase enzyme to the phytate substrate by chelation of metal ions, such as, for example, aluminium, iron or calcium, that are known to associate with phytate in the soil. This hypothesis is consistent with data provided as Example 3 herein which show that organic acids
25 increase the availability of organic P substrates and makes them more amendable to dephosphorylation by phytase.

According to this embodiment, the chemistry of the soil around the root may be altered in accordance with the present invention by any means known to those
30 skilled in the art, including the application of organic acids to the soil or growth medium, or the addition of agents known to those skilled in the art that chelate metals but not phosphorus.

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However, a particularly preferred means comprises expressing an organic acid biosynthetic enzyme in the roots of the plant so as to increase the intracellular level of organic acids, and the subsequent efflux of organic acids from the root.

5 Preferably, the expression of the organic acid biosynthetic enzyme is targeted to the same cells in which the phytase gene is expressed, to optimise the local extracellular concentrations of available phosphorus in that region of the root which is involved in phosphate uptake.

P

10 referably, the organic acid biosynthesis enzyme is citrate synthase. Expression of citrate synthase may be increased in the region around the root by ectopically-expressing a citrate synthase-encoding nucleic acid molecule in the root under the control of a root-specific promoter sequence or constitutive promoter sequence as described herein, and preferably, targeting the citrate synthase polypeptide product

15 of such expression to the root surface.

As will be apparent to those skilled in the art, the ectopic expression of citrate synthase and targeting of the citrate synthase polypeptide to the root surface may be performed in a similar manner to the expression and secretion of the phytase

20 polypeptide, in accordance with the description provided herein. Preferably, in the performance of this embodiment of the invention, the nucleic acid molecules encoding phytase and the organic acid biosynthesis enzyme are placed operably in connection with different promoter sequences, to minimise competition therebetween for nuclear transcription factors, which competition may reduce

25 expression of one or other structural gene.

Alternatively or in addition, the chemistry of the soil or other growth medium is increased by expressing an organic acid transporter polypeptide in the roots of the plant for a time and under conditions sufficient for the rate or amount of organic acid

30 outside the root to increase.

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A further aspect of the present invention clearly provides a gene construct or vector to facilitate the ectopic expression and/or maintenance of the phytase protein-encoding sequence and promoter in a plant cell, tissue or organ.

5 It will be apparent from the preceding statements that the gene construct of the invention will at least comprise a phytase protein-encoding sequence, optionally further comprising nucleotide sequences encoding a secretory signal sequence operable in plant cells, and a promoter sequence operable in the root cells of a plant operably connected thereto.

10

The present invention clearly encompasses genetic constructs that further comprise a nucleotide sequence that encodes an organic acid biosynthesis enzyme, in particular citrate synthase, placed operably under the control of a further root-operable or constitutive promoter sequence.

15

Additionally, the gene construct of the present invention may further comprise one or more terminator sequences.

20 The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3'-non-translated DNA sequences containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3'-end of a primary transcript. Terminators active in cells derived from viruses, yeasts, moulds, bacteria, insects, birds, mammals and plants are known and described in the literature. They may be isolated from
25 bacteria, fungi, viruses, animals and/or plants.

TABLE 2
EXEMPLARY PROMOTERS FOR USE IN THE PERFORMANCE OF THE PRESENT INVENTION

GENE SOURCE	EXPRESSION PATTERN	REFERENCE
root-expressible genes	roots	Tingey et al. (1987); An et al. (1988);
tobacco auxin-inducible gene	root tip	Van der Zaal et al. (1991)
β -tubulin	root	Oppenheimer et al. (1988)
tobacco root-specific genes	root	Conkling et al. (1990)
<i>B. napus</i> G1-3b gene	root	United States Patent No 5, 401, 836
SbPRP1	roots	Suzuki et al. (1993)
AtPRP1; AtPRP3	roots; root hairs	http://salus.medium.edu/mmq/terney/html
RD2 gene	root cortex	http://www2.cnsu.edu/ncsu/research
TobRB7 gene	root vasculature	http://www2.cnsu.edu/ncsu/research
Actin	constitutive	
CaMV 35S	constitutive	Odell et al. (1985)
CaMV 19S	constitutive	
Octopine synthase (OCS)	constitutive	Koncz et al., (1984)
Nopaline Synthase (NOS)	constitutive	Depicker et al., (1982)
gos2	constitutive	de Pater et al. (1992)
UBQ1	constitutive	Callis et al., (1990)

INDUCIBLE PROMOTERS	INDUCTANT	REFERENCE
P5CS (delta(1)-pyrroline-5-carboxylate synthase)	salt, water	Zhang <i>et al</i> (1997)
cor15a	cold	Hajela <i>et al</i> (1990)
cor15b	cold	Wilhelm <i>et al</i> (1993)
cor15a (-305 to +78 nt)	cold, drought	Baker <i>et al</i> (1994)
rd29	salt, drought, cold	Kasuga <i>et al</i> (1999)
heat shock proteins, including artificial promoters containing the heat shock element (HSE)	heat	Barros <i>et al</i> (1992); Marrs <i>et al</i> (1993); Schoff <i>et al</i> (1989)
smHSP (small heat shock proteins)	heat	Waters <i>et al.</i> (1989)
wcs120	cold	Oullet <i>et al</i> (1998)
ci7	cold	Kirch <i>et al</i> (1997)
Adh	cold, drought, hypoxia	Dolferus <i>et al</i> (1994)
pws18	water, salt and drought	Joshee <i>et al</i> (1998)
ci21A	cold	Schneider <i>et al</i> (1997)
Trg-31	drought	Chaudhury <i>et al</i> (1995)
osmotin	osmotic stress	Raghothama <i>et al</i> (1993)

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Examples of terminators particularly suitable for use in the gene constructs of the present invention include the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene terminator, the *Agrobacterium tumefaciens* octopine synthase (OCS) gene terminator sequence, the Cauliflower mosaic virus (CaMV) 35S gene terminator sequence, the *Oryza sativa* ADP-glucose pyrophosphorylase terminator sequence (t3'Bt2), the *Zea mays zein* gene terminator sequence, the *rbcs-1A* gene terminator, and the *rbcs-3A* gene terminator sequences, amongst others.

Those skilled in the art will be aware of additional promoter sequences and terminator sequences which may be suitable for use in performing the invention. Such sequences may readily be used without any undue experimentation.

The gene constructs of the invention may further include an origin of replication sequence which is required for maintenance and/or replication in a specific cell type, for example a bacterial cell, when said gene construct is required to be maintained as an episomal genetic element (eg. plasmid or cosmid molecule) in said cell. Preferred origins of replication include, but are not limited to, the *f1*-ori and *colE1* origins of replication.

The gene construct may further comprise a selectable marker gene or genes that are functional in a cell into which said gene construct is introduced.

As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a gene construct of the invention or a derivative thereof.

Suitable selectable marker genes contemplated herein include the ampicillin resistance (Amp^r), tetracycline resistance gene (Tc^r), bacterial kanamycin resistance gene (Kan^r), phosphinothricin resistance gene, neomycin phosphotransferase gene (*npfII*), hygromycin resistance gene, β -glucuronidase

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(GUS) gene, chloramphenicol acetyltransferase (CAT) gene, green fluorescent protein (*gfp*) gene (Haseloff *et al*, 1997), and luciferase gene, amongst others.

In fact, the phytase protein-encoding sequence may also be used as a selectable marker gene as defined herein, by virtue of the improved phosphorus nutrition of plants secreting phytase from their roots in accordance with the inventive method, including their ability to grow on phytate-containing media. Accordingly, the present invention clearly encompasses the selection of transformed plants by their ability to regenerate on media having phytate as the source of phosphorus.

In a further preferred embodiment, the present invention provides a method of modifying the phosphorus nutrition of a plant comprising:

- (i) introducing to a plant cell, tissue or organ a gene construct or vector comprising a nucleotide sequence that encodes phytase in a secretable form, operably in connection with a promoter sequence capable of conferring expression in the roots of a plant;
- (ii) regenerating a whole plant therefrom; and
- (ii) expressing said phytase in the root or one or more of said cells or tissues thereof such that it is secretable to the surface of the root.

A further aspect of the invention provides a transformed plant ectopically-expressing phytase in secretable form, preferably as an in-frame fusion with a secretable signal sequence.

Means for introducing recombinant DNA into bacterial cells, yeast cells, or plant, insect, fungal (including mould), avian or mammalian tissue or cells include, but are not limited to, transformation using CaCl_2 and variations thereof, in particular the method described by Hanahan (1983), direct DNA uptake into protoplasts (Krens *et al*, 1982; Paszkowski *et al*, 1984), PEG-mediated uptake to protoplasts (Armstrong *et al*, 1990), electroporation (Fromm *et al*, 1985), microinjection of DNA (Crossway *et al*, 1986), microparticle bombardment of tissue explants or cells (Christou *et al*,

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1988; Sanford *et al.*, 1987; Finer and McMullen, 1990; Finer *et al.*, 1992; Sanford *et al.*, 1993; Karunaratne *et al.*, 1996; and Abedinia *et al.*, 1997), vacuum-infiltration of tissue with nucleic acid, or T-DNA-mediated transfer from *Agrobacterium* to the plant tissue (An *et al.* 1985; Herrera-Estrella *et al.*, 1983a; 1983b; 1985).

5

For example, the transformed plants can be produced by the method of *in planta* transformation method using *Agrobacterium tumefaciens* (Bechtold *et al.*, 1993; Clough *et al.*, 1998), wherein *A. tumefaciens* is applied to the outside of the developing flower bud and the binary vector DNA is then introduced to the developing microspore and/or macrospore and/or the developing seed, so as to produce a transformed seed. Those skilled in the art will be aware that the selection of tissue for use in such a procedure may vary, however it is preferable generally to use plant material at the zygote formation stage for *in planta* transformation procedures.

15

Alternatively, microparticle bombardment of cells or tissues may be used, particularly in cases where plant cells are not amenable to transformation mediated by *A. tumefaciens*. In such procedures, microparticle is propelled into a cell to produce a transformed cell. Any suitable ballistic cell transformation methodology and apparatus can be used in performing the present invention. Exemplary apparatus and procedures are disclosed by Stomp *et al.* (U.S. Patent No. 5,122,466) and Sanford and Wolf (U.S. Patent No. 4,945,050). When using ballistic transformation procedures, the genetic construct may incorporate a plasmid capable of replicating in the cell to be transformed.

25

Examples of microparticles suitable for use in such systems include 1 to 5 μm gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

30

A whole plant may be regenerated from the transformed or transfected cell, in accordance with procedures well known in the art. Plant tissue capable of

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subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a gene construct of the present invention and a whole plant regenerated therefrom. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

10 The term "organogenesis", as used herein, means a process by which shoots and roots are developed sequentially from meristematic centres.

The term "embryogenesis", as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes.

The generated transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plant may be selfed to give homozygous second generation (or T2) transformant, and the T2 plants further propagated through classical breeding techniques.

The generated transformed organisms contemplated herein may take a variety of forms. For example, they may be chimeras of transformed cells and non-transformed cells; clonal transformants (e.g., all cells transformed to contain the expression cassette); grafts of transformed and untransformed tissues (e.g., in plants, a transformed root stock grafted to an untransformed scion).

The present invention is applicable to any plant, in a particular monocotyledonous plants or dicotyledonous plant including fodder or forage legume, companion plant, food crop, tree, shrub, or ornamental selected from the list comprising *Acacia spp.*,

- Acer spp.*, *Actinidia spp.*, *Aesculus spp.*, *Agathis australis*, *Albizia amara*, *Alsophila tricolor*, *Andropogon spp.*, *A. thaliana*, *Arachis spp.*, *Areca catechu*, *Astelia fragrans*, *Astragalus cicer*, *Baikiaea plurijuga*, *Betula spp.*, *Brassica spp.*, *Bruguiera gymnorhiza*, *Burkea africana*, *Butea frondosa*, *Cadaba farinosa*, *Calliandra spp.*,
5 *Camellia sinensis*, *Canna indica*, *Capsicum spp.*, *Cassia spp.*, *Centroema pubescens*, *Chaenomeles spp.*, *Cinnamomum cassia*, *Coffea arabica*, *Colophospermum mopane*, *Coronilla varia*, *Cotoneaster serotina*, *Crataegus spp.*, *Cucumis spp.*, *Cupressus spp.*, *Cyathea dealbata*, *Cydonia oblonga*, *Cryptomeria japonica*, *Cymbopogon spp.*, *Cynthea dealbata*, *Cydonia oblonga*, *Dalbergia*
10 *monetaria*, *Davallia divaricata*, *Desmodium spp.*, *Dicksonia squarosa*, *Diheteropogon amplexans*, *Dioclea spp.*, *Dolichos spp.*, *Dorycnium rectum*, *Echinochloa pyramidalis*, *Ehrartia spp.*, *Eleusine coracana*, *Eragrestis spp.*, *Erythrina spp.*, *Eucalyptus spp.*, *Euclea schimperii*, *Eulalia villosa*, *Fagopyrum spp.*, *Feijoa sellowiana*, *Fragaria spp.*, *Flemingia spp.*, *Freycinetia banksii*, *Geranium thunbergii*,
15 *Ginkgo biloba*, *Glycine javanica*, *Gliricidia spp.*, *Gossypium hirsutum*, *Grevillea spp.*, *Guibourtia coleosperma*, *Hedysarum spp.*, *Hemarthia altissima*, *Heteropogon contortus*, *Hordeum vulgare*, *Hyparrhenia rufa*, *Hypericum erectum*, *Hyperthelia dissoluta*, *Indigo incarnata*, *Iris spp.*, *Leptarrhena pyrolifolia*, *Lespedeza spp.*, *Lettuca spp.*, *Leucaena leucocephala*, *Loudetia simplex*, *Lotonus bainesii*, *Lotus spp.*,
20 *Macrotyloma axillare*, *Malus spp.*, *Manihot esculenta*, *Medicago sativa*, *Metasequoia glyptostroboides*, *Musa sapientum*, *Nicotianum spp.*, *Onobrychis spp.*, *Ornithopus spp.*, *Oryza spp.*, *Peltophorum africanum*, *Pennisetum spp.*, *Persea gratissima*, *Petunia spp.*, *Phaseolus spp.*, *Phoenix canariensis*, *Phormium cookianum*, *Photinia spp.*, *Picea glauca*, *Pinus spp.*, *Pisum sativum*, *Podocarpus totara*, *Pogonarthria*
25 *fleckii*, *Pogonarthria squarrosa*, *Populus spp.*, *Prosopis cineraria*, *Pseudotsuga menziesii*, *Pterolobium stellatum*, *Pyrus communis*, *Quercus spp.*, *Rhaphiolepis umbellata*, *Rhopalostylis sapida*, *Rhus natalensis*, *Ribes grossularia*, *Ribes spp.*, *Robinia pseudoacacia*, *Rosa spp.*, *Rubus spp.*, *Salix spp.*, *Schyzachyrium sanguineum*, *Sciadopitys verticillata*, *Sequoia sempervirens*, *Sequoiadendron*
30 *giganteum*, *Sorghum bicolor*, *Spinacia spp.*, *Sporobolus fimbriatus*, *Stiburus alopecuroides*, *Stylosanthos humilis*, *Tadehagi spp.*, *Taxodium distichum*, *Themeda*

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triandra, *Trifolium* spp., *Triticum* spp., *Tsuga heterophylla*, *Vaccinium* spp., *Vicia* spp., *Vitis vinifera*, *Watsonia pyramidata*, *Zantedeschia aethiopica*, *Zea mays*, rice, straw, amaranth, onion, asparagus, sugar cane, soybean, sugarbeet, sunflower, carrot, celery, cabbage, canola, tomato, potato, lentil, flax, broccoli, oilseed rape, cauliflower, brussel sprout, artichoke, okra, squash, kale, collard greens, and tea, amongst others, or the seeds of any plant specifically named above or a tissue, cell or organ culture of any of the above species.

Preferably, the plant is a plant that is capable of being transfected or transformed with a genetic sequence, or which is amenable to the introduction of a protein by any art-recognised means, such as microprojectile bombardment, microinjection, *Agrobacterium-mediated* transformation, protoplast fusion, protoplast transformation, *in planta* transformation, or electroporation, amongst others.

In a particularly preferred embodiment, the transformed plant is *A. thaliana* or subterranean clover. As will be known to those skilled in the art, the provision of both transformed species is sufficient to enable the enhancement of phosphorus nutrition in any plant species using the inventive method described herein.

This aspect of the invention further extends to plant cells, tissues, organs and plants parts, propagules and progeny plants of the primary transformed or transfected cells, tissues, organs or whole plants that also comprise the introduced isolated nucleic acid molecule or gene construct comprising same, and, as a consequence, exhibit similar phenotypes to the primary transformants/transfectants or at least are useful for the purpose of replicating or reproducing said primary transformants/transfectants.

A further aspect of the invention provides an isolated nucleic acid molecule comprising a nucleotide sequence which encodes or is complementary to a nucleotide sequence which encodes a phytase peptide, oligopeptide, polypeptide, protein or enzyme having at least about 93% nucleotide sequence identity to the

Aspergillus niger *PhyA-2* gene sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 9 or a complementary nucleotide sequence thereto.

Preferably, the percentage identity to SEQ ID NO: 1 or SEQ ID NO: 9 is at least about 95%, more preferably at least about 97%, and still more preferably at least about 99%. In a particularly preferred embodiment, the isolated nucleic acid molecule of the invention comprises or contains the nucleotide sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 9 or a fragment thereof that encodes an enzymically-functional phytase peptide, oligopeptide or polypeptide.

In determining whether or not two nucleotide or amino acid sequences fall within defined percentage identity or similarity limits referred to herein, those skilled in the art will be aware that it is necessary to conduct a side-by-side comparison. In such comparisons or alignments, differences will arise in the positioning of non-identical residues depending upon the algorithm used to perform the alignment. In the present context, references to percentage identities between two or more sequences shall be taken to refer to the number of identical and similar residues respectively, between said sequences as determined using any standard algorithm known to those skilled in the art. In particular, nucleotide and/or amino acid identities can be calculated using the GAP programme of the Computer Genetics Group, Inc., University Research Park, Madison, Wisconsin, United States of America (Devereaux *et al*, 1984), which utilizes the algorithm of Needleman and Wunsch (1970) or alternatively, the CLUSTAL W algorithm of Thompson *et al* (1994) for multiple alignments, to maximise the number of identical/similar residues and to minimise the number and/or length of sequence gaps in the alignment.

Alternatively or in addition, the present invention encompasses those phytase-encoding nucleotide sequences that are capable of hybridising under high stringency hybridisation conditions to SEQ ID NO: 1 or SEQ ID NO: 9 or a complementary nucleotide sequence thereto, but not including the *PhyA-1* gene sequence.

For the purposes of defining the level of stringency, those skilled in the art will be aware that several different hybridisation conditions may be employed. As used herein, a high stringency may comprise a standard reaction buffer used in a polymerase chain reaction (PCR) to anneal an oligonucleotide primer to template DNA at temperatures higher than 42°C, or alternatively, a standard DNA/DNA hybridisation and/or wash carried out in 0.1xSSC buffer, 0.1% (w/v) SDS at a temperature of at least 65°C, or equivalent annealing/hybridisation conditions.

As will be known to those skilled in the art, the stringency is increased by reducing the concentration of SSC buffer, and/or increasing the concentration of SDS in a standard hybridisation, and/or increasing the temperature of the annealing/hybridisation of PCR or a standard hybridisation, and/or increasing the temperature of the wash in a standard hybridisation. Conditions for hybridisations and washes are well understood by one normally skilled in the art. For the purposes of clarification (to parameters affecting hybridisation between nucleic acid molecules), reference is found in pages 2.10.8 to 2.10.16. of Ausubel *et al.* (1987), which is herein incorporated by reference.

As will be known to those skilled in the art, the specificity of PCR may also be increased by reducing the number of cycles, or the time per cycle, or by the use of specific PCR formats, such as, for example, a nested PCR, a format that is well-known to those skilled in the art. For the purposes of clarification of the parameters affecting the specificity of PCR, reference is made herein to McPherson *et al.* (1991) which is incorporated by way of reference.

Particularly preferred variants of the *A. niger PhyA-2* gene exemplified herein comprise degenerate nucleotide sequences (i.e. homologues) that encode the amino acid sequence set forth in SEQ ID NO: 2.

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Preferably, the isolated nucleic acid molecule encodes a phytase polypeptide, protein or enzyme as an in-frame fusion with a secretory signal peptide, in particular the carrot extensin signal peptide.

5 Homologues, analogues and derivatives of the phytase-encoding nucleotide sequence of the present invention may be obtained by any standard procedure known to those skilled in the art, such as by nucleic acid hybridization (Ausubel *et al*, 1987), polymerase chain reaction (McPherson *et al*, 1991) screening of expression libraries using antibody probes (Huynh *et al*, 1985), and the invention encompasses
10 all such homologues, analogues and derivatives falling within the above-mentioned sequence identity and/or hybridisation limitations.

In nucleic acid hybridizations, genomic DNA, mRNA or cDNA or a part of fragment thereof, in isolated form or contained within a suitable cloning vector such as a
15 plasmid or bacteriophage or cosmid molecule, is contacted with a hybridization-effective amount of a nucleic acid probe derived from SEQ ID NO: 1 for a time and under conditions sufficient for hybridization to occur and the hybridized nucleic acid is then detected using a detecting means.

20 Detection is performed preferably by labelling the probe with a reporter molecule capable of producing an identifiable signal, prior to hybridization. Preferred reporter molecules include radioactively-labelled nucleotide triphosphates and biotinylated molecules.

25 Preferably, variants of the *A. niger* *PhyA-2* gene exemplified herein, including genomic equivalents, are isolated by hybridisation under high stringency conditions, to the probe.

In the polymerase chain reaction (PCR), a nucleic acid primer molecule comprising
30 at least about 14 nucleotides in length derived from the *A. niger* *PhyA-2* gene is hybridized to a nucleic acid template molecule and specific nucleic acid molecule

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copies of the template are amplified enzymatically as described in McPherson *et al*, (1991), which is incorporated herein by reference.

In expression screening of cDNA libraries or genomic libraries, protein- or peptide-
5 encoding regions are placed operably under the control of a suitable promoter sequence in the sense orientation, expressed in a prokaryotic cell or eukaryotic cell in which said promoter is operable to produce a peptide or polypeptide, screened with a monoclonal or polyclonal antibody molecule or a derivative thereof against one or more epitopes of a phytase polypeptide and the bound antibody is then
10 detected using a detecting means, essentially as described by Huynh *et al* (1985) which is incorporated herein by reference. Suitable detecting means according to this embodiment include ¹²⁵I-labelled antibodies or enzyme-labelled antibodies capable of binding to the first-mentioned antibody, amongst others.

15 A still further aspect of the present invention provides an isolated or recombinant phytase polypeptide selected from the group consisting of:

- (i) a phytase polypeptide having at least 95% amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO: 2;
- (ii) a phytase polypeptide encoded by a nucleotide sequence having at
20 least 93% identity to SEQ ID NO: 1;
- (iii) fragments of (i) or (ii) that possess phytase enzyme activity;
- (iv) in-frame fusion polypeptides comprising (i) and/or (ii) and/or (iii) linked to a secretory signal peptide, in particular the carrot extensin secretory signal peptide.

25

It will be apparent from the preceding description that a recombinant phytase polypeptide or an in-frame fusion polypeptide comprising same may be produced by standard means by expressing a phytase-encoding nucleotide sequence operably under the control of a suitable promoter sequence in a host cell for a time and under
30 conditions sufficient for translation to occur. Such expression may be carried out in a prokaryotic cell, such as, for example, a bacterial cell. Alternatively, such expression

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may be performed in a eukaryotic cell such as an insect cell, mammalian cell, plant cell, fungal cell, or yeast cell, amongst others. In any case, unless the sense molecule is expressed under the control of a strong universal promoter, it is important to select a promoter sequence which is capable of regulating expression in the cell comprising the said nucleic acid molecule in an expressible format. Persons skilled in the art will be in a position to select appropriate promoter sequences for expression of the sense molecule without undue experimentation.

Examples of promoters useful in performing this embodiment include the CaMV 35S promoter, NOS promoter, octopine synthase (OCS) promoter, *A. thaliana* SSU gene promoter, napin seed-specific promoter, P₃₂ promoter, BK5-T *imm* promoter, *lac* promoter, *tac* promoter, phage lambda λ_L or λ_R promoters, CMV promoter (U.S. Patent No. 5,168,062), T7 promoter, lacUV5 promoter, SV40 early promoter (U.S. Patent No. 5,118,627), SV40 late promoter (U.S. Patent No. 5,118,627), adenovirus promoter, baculovirus P10 or polyhedrin promoter (U.S. Patent Nos. 5,243,041, 5,242,687, 5,266,317, 4,745,051 and 5,169,784), and the like. In addition to the specific promoters identified herein, cellular promoters for so-called housekeeping genes are useful.

In a preferred embodiment, the recombinant phytase polypeptide is provided in a sequencably-pure format or a pure format substantially free of conspecific proteins.

By "sequencably pure" is meant that the subject polypeptide or a homologue, analogue, derivative or epitope thereof is purified sufficiently to facilitate amino acid sequence determination.

Preferably, said polypeptide or a homologue, analogue, derivative or epitope is at least about 20% pure, more preferably at least about 40% pure, even more preferably at least about 60% pure and even more preferably at least about 80% pure or 95% pure on a weight basis.

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For the purposes of describing the present invention in more detail, a plasmid comprising *A. niger* *PhyA-2* gene as an in-frame fusion with the carrot extensin secretion signal-encoding nucleotide sequence, and operably in connection with the CaMV 35S promoter, was deposited on 23 September, 1999, with the Australian
5 Government Analytical Laboratories (AGAL) at 1, Suakin Street Pymble, New South Wales 2073, Australia, under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, and accorded AGAL Accession No. NM99/06795. Accordingly, the presently-described invention clearly extends to the use of the deposited plasmid
10 and/or the phytase-encoding portion thereof or variants thereof, with or without the secretory signal-encoding portion of said plasmid, in accordance with the scope of each and every embodiment described herein.

The present invention is further described with reference to the following non-limiting
15 Examples and to the drawings.

EXAMPLE 1

Ectopic expression of phytase under control of the CaMV 35S promoter

20 I. The *PhyA-2* gene

PCR primers PHYF2 and PHYR3 were used to amplify a derivative of the *PhyA-1* gene from genomic DNA isolated from *Aspergillus niger*, strain ATCC9029. The sequence of the PCR primers (which also contained cloning sites for *EcoRI* and *Clal*, respectively; underlined lower case) were as follows:

25 Forward(PHYF2): cgcgaattcATGCTGGCAGTCCCCGCCTCG (SEQ ID NO: 13); and
Reverse(PHYR3): ggcatcgatCTAAGCAAAACACTCCGC (SEQ ID NO: 14)

The amplified *PhyA-2* gene is a modified version of the *PhyA-1* gene that is functional in plants, and contains no leader sequence or first intron; a "new" ATG
30 translation start in the open reading frame, immediately prior to, and in frame with, the nucleotide sequence encoding the mature phytase polypeptide. The sequence

of the reverse primer resulted in the TAG translation stop codon being identical to that in the published *A. niger* *PhyA-1* gene. The amplified gene has been designated "PhyA-2".

- 5 The *PhyA-2* gene has approximately 92% DNA sequence identity to the *PhyA-1* gene. Additionally, there is about 95% amino acid sequence identity between *PhyA-1* and *PhyA-2* polypeptides (Figures 1 and 2).

The nucleotide sequence of the *PhyA-2* gene is set forth in SEQ ID NO: 1, and the derived amino acid sequence encoded by the *PhyA-2* gene is set forth in SEQ ID
10 NO: 2.

II. The *PhyA-1* gene

The *PhyA-1* gene was originally obtained from Dr Mullaney (United States Department of Agriculture) as a 7.016 kb plasmid (pMD4.21), containing a 2.7 kb
15 *SphI* clone of the *Aspergillus ficuum* strain NRRL3135 (now termed *A. niger*) *PhyA-1* gene, cloned into the plasmid vector pBR322.

The 2.7 kb insert contained a genomic clone of the *PhyA-1* gene with 5' and 3' flanking sequences, including an *Aspergillus* 5' leader sequence and an intron (102
20 bp) upstream of the coding region for the mature protein (Genbank Accession No. M94550; Van Hartingsveldt *et al.*, 1993).

Modifications were made to the *PhyA-1* gene, to delete the leader sequence and the intron, using PCR. These modifications introduced a "new" ATG translation start in
25 the open reading frame, immediately prior to, and in frame with, the nucleotide sequence for the mature peptide. The sequence of the reverse primer resulted in the TAG translation stop codon being identical to that in the published *A. niger* *PhyA-1* gene.

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The sequence of the PCR primers (which also contained cloning sites for *EcoRI* and *Clal*, respectively; underlined lower case) to obtain a modified version of the gene that is functional in plants were as follows:

- 5 Forward(PHYF2): cgcgaattcATGCTGGCAGTCCCCGCCTCG (SEQ ID NO: 13);
and
Reverse(PHYR3): ggcatcgatCTAAGCAAACACTCCGC (SEQ ID NO: 14)

- 10 The nucleotide sequence of the modified *PhyA-1* gene is set forth in SEQ ID NO: 3,
and the derived amino acid sequence encoded by this *PhyA-2* gene is set forth in
SEQ ID NO: 4.

III. The Carrot extensin secretory signal sequence.

- 15 Nucleotide sequences encoding the secretory signal sequence of the carrot extensin
gene was amplified using PCR, from plasmid pSEGON, obtained from D. Llewellyn,
Commonwealth Scientific and Industrial Research Organisation, Canberra, Australia,
which plasmid contains nucleotide sequences encoding the extensin signal peptide
upstream of a glucose oxidase-encoding gene.

- 20 The PCR primers used to amplify the extensin secretory signal sequence were as
follows, wherein cloning sites in the primers are underlined and in lower case:

Forward: gcgtctagagaattcATGGGAAGAATTGCTAG (SEQ ID NO: 15); and
Reverse: cgcggatccgcggccgcAGCTGTGGTTTCGGAAGC (SEQ ID NO: 16).

- 25 The amplified product was first subcloned as a *XbaI/BamHI* fragment into plasmid
pBSIIKS (Stratagene, Palo Alto, California, USA). The amplified fragment was 99 bp
in length and codes for 33 amino acids. The sequence of the leader is set forth in
SEQ ID NO: 5.

30 IV. Extensin/Phytase Gene Fusion

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In-frame" fusions between the extensin secretory signal-encoding sequence and both the *PhyA-1* and *PhyA-2* genes were generated by ligation between the *NotI* site located at the 3' end of the extension secretory signal-encoding sequence and the *EcoRI* site at the 5' end of the *PhyA-1* and *PhyA-2* genes, respectively. This was achieved by generating blunt (ie., flushed) ends of the restriction enzymes sites prior to ligation.

The junction sequence generated from the extensin/phytase fusion comprised the nucleotide sequence 5'-ACGCTGCCATGCTGGCA-3' (SEQ ID NO: 17), encoding the junction amino acid sequence Thr-Ala-Ala-Met-Leu-Ala (SEQ ID NO: 18), which amino acid sequence comprises two codons derived from extensin, an inserted alanine (encoded by the *NotI-EcoRI* fusion), and three codons derived from the phytase genes (*PhyA-1* or *PhyA-2* as appropriate).

Without being bound by any theory or mode of action, the Alanine residue at the extensin::phytase junction provides a suitable site for protease cleavage of the precursor polypeptide to remove the extensin secretory signal sequence.

The extensin/phytase in-frame fusions were generated and verified in plasmid pBSIIKS.

The nucleotide sequence of the *extensin::PhyA-2* chimeric gene is set forth in SEQ ID NO: 9, and the amino acid sequence encoded therefor is set forth in SEQ ID NO: 10. The nucleotide sequence of the *extensin::PhyA-1* chimeric gene is set forth in SEQ ID NO: 11, and the amino acid sequence encoded therefor is set fourth in SEQ ID NO: 12.

V. Construction of vectors for plant transformation

Four gene expression cassettes were produced, comprising the *PhyA-1* gene, chimeric *extensin::PhyA-1* gene, *PhyA-2* gene, and chimeric *extensin::PhyA-2* gene, by subcloning these genes, as *EcoRI-KpnI* fragments, from their plasmid pBSIIKS

vector (see above) into plasmid pART7 (Figure 4), which contains a CaMV 35S promoter and ocs terminator.

Accordingly, in the resultant plasmid constructs, the phytase genes were placed in operable connection with the CaMV 35S promoter sequence, to produce the plasmids:

1. 35S+PhyA-1+ocs;
2. 35S+extensin::PhyA-1+ocs;
3. 35S+PhyA-2+ocs; and
- 10 4. 35S+extensin::PhyA-2+ocs.

The four phytase transgenes were then subcloned as *NotI* fragments into the binary vector pBS389 (Figure 3). This vector is suitable for plant transformation and contains the selectable marker *np11* under control of the SCSV Sc1 promoter and Sc3 terminator sequences. The phytase transgenes were cloned such that their orientation (determining the direction of transcription) was the same as the orientation as the selectable marker.

VI. Plant transformation

The pBS389 vectors containing the various phytase gene constructs were transferred to *Agrobacterium tumefaciens* strain AGL1 using standard, tri-parental mating techniques. These strains were used to transform tobacco, *A. thaliana* and subterranean clover using published protocols. Transformed plants of all three species were generated and have been verified to contain the various phytase transgenes by selection on Kanamycin, PCR and by Southern blot analysis using the *PhyA-1* gene as a hybridisation probe.

- Figure 12 shows a representative Southern blot hybridisation to the *PhyA-1* nucleotide sequence under high stringency conditions, of DNAs derived from several lines of *A. thaliana* (C24 ecotype) plants generated using plasmid pBS389, or the *PhyA-2* gene (SEQ ID NO: 1) in plasmid pBS389 (no leader sequence), or plasmid pAER04 (Figure 6), or plasmid containing the *PhyA-1* gene (SEQ ID NO: 3) in plasmid pBS389 (no leader sequence); or plasmid pAER02 (Figure 5). Data indicate the presence of the *PhyA-1*-hybridising signal only in plants produced using the *PhyA-1*-containing or *PhyA-2*-containing gene constructs.

VII. Utilization of organic phosphorus substrates by non-transformed plants

- When grown under sterile conditions, wild-type (i.e. non-transformed) *A. thaliana* plants were able to access phosphorus from a range of organic phosphorus substrates, and their growth and phosphorus nutrition were comparable to that for plants supplied with inorganic phosphate. By contrast, wild-type plants were unable to acquire P from phytate. When supplied with phytate, a lower shoot dry weight was observed, and the total phosphorus content of the shoots was only 9.3% of the total phosphorus content of plants supplied with inorganic phosphate as Na_2HPO_4 , and not significantly different ($P < 0.05$) from plants grown in the absence of external phosphorus.
- Similarly, shoot phosphorus concentrations of non-transformed plants supplied with phytate, or alternatively, grown without an external phosphorus source were

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significantly lower and these plants exhibited markedly lower shoot to root ratios than plants grown on inorganic phosphate as Na_2HPO_4 .

5 In general, no major differences in root dry weights were observed across the various phosphorus treatments, although lower root mass was evident for plants grown in the absence of phosphorus.

The observed phenotype of plants grown on phytate are indicative of a phosphorus deficiency.

10

Total extracellular acid phosphomonoesterase activities of between 13.6 and 25.5 mU mg^{-1} dry weight were measured for roots of *A. thaliana* plants, with no significant differences ($P < 0.05$) occurring between phosphorus-deficient plant grown on phytate or in the absence of phosphorus, and phosphorus-sufficient plants grown on inorganic phosphate as Na_2HPO_4 . Similarly, no differences were observed in total acid phosphomonoesterase in crude extracts prepared from whole roots. In these

15

VIII. Analysis of transformed plants

A) Production of phytase-encoding mRNA

Northern blot hybridisations have been conducted to confirm ectopic expression of the introduced phytase genes transgenic tobacco, *A. thaliana* and subterranean

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Figure 11 shows a representative northern blot hybridisation to the *PhyA-1* nucleotide sequence, of several lines of transgenic *A. thaliana* (C24 ecotype) plants carrying the *PhyA-2* gene (SEQ ID NO: 1) in plasmid pBS389 (no leader sequence), or plasmid pAER04 (Figure 6), or plasmid containing the *PhyA-1* gene (SEQ ID NO: 3) in plasmid pBS389 (no leader sequence); or plasmid pAER02 (Figure 5). Data

25

obtained indicated that, in most cases, highest levels of phytase-encoding mRNA were present in plants that contain the phytase genes (*PhyA-1* or *PhyA-2*) fused to the extensin secretory signal-encoding nucleotide sequence, suggesting that these sequences may also increase mRNA stability.

5

Figure 13 shows a northern blot experiment showing ectopic expression of phytase genes in five independent lines of transgenic *Trifolium subterraneum* (subterranean clover, cultivar Dalkeith; T₀ generation), containing plasmid pAER02 (Figure 5). Data indicate a positive correlation between copy number of the introduced transgene and the level of expression of the chimeric extensin::*PhyA-2* transgene.

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b) Phytase enzyme activity

(i) Transformed tobacco plants

Phytase enzyme assays were performed using leaf material derived from transformed tobacco plants.

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The phytase activity in leaf extracts of control plants was 0.24 nkat phytase g⁻¹ fresh wt (0.04 nkat phytase mg⁻¹ protein), compared to 30.4 nkat g⁻¹ fresh wt (6.2 nkat phytase mg⁻¹ protein) for transformed plants containing the *extensin::PhyA-1* gene construct, representing a 130-fold increase in enzyme activity. Similar results are obtained for phytase assays of roots.

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(ii) Transformed subterranean clover plants

Phytase enzyme activity increased in the leaves and roots of transgenic subterranean clover that contain the introduced phytase gene constructs. In particular, the phytase activity in the shoots of primary transformants of subterranean clover generated from tissue culture explant material transformed with plasmid pAER02 was approximately 24-fold that observed in the shoots of control plants transformed with the vector pBS389 (Table 3). In contrast, the phytase activity of transformed plants carrying the *PhyA-1* gene without the extensin signal peptide,

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was not significantly different from that of the control plants transformed with pBS389.

TABLE 3

5 **Phytase activity of transgenic subterranean clover plants: T0 generation**

Construct	Number of plants	Phytase activity (mU phytase g ⁻¹ fresh wt)		
		mean	range	
control*	3	100.1	35.5	- 190.5
<i>PhyA-1</i>	9	76.8	38.7	- 96.9
<i>ext::PhyA-1</i>	20	2443.5	413.3	- 4346.0

15

* Control plants were transformed with the binary vector pBS389. The phytase containing lines were transformed with derivatives of pBS389 containing *PhyA-1* either without (*PhyA-1*) or with (*ext::PhyA-1*) the extracellular-targeting sequence from the carrot extensin gene.

20

The high phytase activity of the T0 plant lines expressing the extensin::PhyA-1 fusion polypeptide was transmissible to the T1 generation, and, for the line designated "iv" there was a 3:1 segregation ratio of high phytase:low phytase, consistent with a single gene insertion in the primary transformant (Table 4). For the line designated "i" there was a 15:1 segregation ratio of high phytase:low phytase, consistent with a double gene insertion in the primary transformant giving rise to that line (Table 4). The number of insertions of the *ex::phyA* gene was confirmed by Southern blot analyses.

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TABLE 4

Phytase activity of transgenic subterranean clover plants: T1 population

5	LINE IV		LINE I	
	plant number	phytase activity (mU phytase g ⁻¹ FW)	plant number	phytase activity (mU phytase g ⁻¹ FW)
10	1	34.3	1	1220.9
	2	1253.2	2	1840.5
	3	110.9	3	1517.6
	4	544.9	4	1995.9
	5	1327.9	5	1247.2
	6	165.5	6	837.5
15	7	813.3	7	1279.5
	8	998.9	8	994.9
	9	990.9	9	991.0
	10	1545.9	10	577.2
	11	238.2	11	861.7
20	12	94.8	12	1961.6
	13	879.9	13	974.7
	14	1247.2	14	226.1
	15	161.5	15	2036.2
	16	1693.2	16	244.2
	17	938.4	17	1461.1
25	18	1495.4	18	750.7
	19	871.8	19	379.4
	20	1245.2	20	1428.8
	control-1 [#]	106.9	control-1	197.7
	control-2	50.5	control-2	60.5

30 [#] Control plants were transformed with the binary vector pBS389. The phytase containing lines were transformed with *PhyA-1* containing the extracellular-targeting sequence (*ext::phyA-1*) from the carrot extensin gene.

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(iii) Transformed *A. thaliana* plants

We determined the phytase enzyme activity in root extracts for two independently (a and b) transformed lines of *A. thaliana* (C24 ecotype) for each transgene referred to herein above. The plants were grown for 36 days on sterile agar (~120 plants per replicate sample) containing 0.8 mM inorganic phosphate as . For P determination, the plants were supplied with an equivalent concentration of P as phytate and were grown for 42 days (3 plants tube⁻¹, Figure 3a-e).

High levels of phytase activity were observed in soluble root extracts prepared from transgenic lines of *A. thaliana* that ectopically-expressed phytase from either the chimeric *ext::PhyA-1* or *ext::PhyA-2* genes (Table 5). Relative to control plants, the phytase activity of roots from lines generated by transformation with the *ext::PhyA-2* and *ext::PhyA-1* chimeric genes were increased by approximately 20- to 25-fold, and by up to 1500-fold, respectively.

In contrast, no difference in phytase activity was observed between control plants, and transformed plants produced by transformation with the *PhyA-1* and *PhyA-2* genes (i.e. without the signal transport peptide-encoding gene sequence attached; Table 5). Notwithstanding the absence of a detectable difference in phytase enzyme activity, we could confirm the expression of mRNA encoding phytase in Northern blot experiments (Figure 11).

TABLE 5.

Phytase activity and P nutrition of transgenic *A. thaliana* plants

Transgenic line*		Root phytase (mU mg ⁻¹ protein)	Total shoot P content† (µg P)	Shoot phosphorus (µg mg ⁻¹ dry wt)
Control*	a	11.4 ^{a§}	6.3 ^a	2.6 ^a
	b	9.3 ^a	7.3 ^a	1.9 ^a
PhyA-2	a	3.9 ^a	6.7 ^a	2.4 ^a
	b	6.6 ^a	6.9 ^a	2.3 ^a
ext::phyA-2	a	224.2 ^b	97.4 ^b	11.2 ^b
	b	432.6 ^b	80.7 ^b	10.2 ^b
PhyA-1	a	14.3 ^a	6.4 ^a	2.0 ^a
	b	11.7 ^a	5.9 ^a	3.0 ^a
ext::phyA-1	a	992.5 ^c	99.1 ^b	10.4 ^b
	b	14,980.4 ^d	81.9 ^b	11.7 ^b

* Control plants were transformed with pBS389.

5 † Across all transgenic lines, the mean phosphorus content of shoots from plants that were grown under identical conditions, but were supplied with inorganic phosphate (P_i), or alternatively, that were grown with no added phosphorus, was 79.9 ± 28.7 and 1.1 ± 0.6 µg, respectively.

10 ‡ Across all transgenic lines, the mean shoot phosphorus content for control plants supplied with P_i, or alternatively, were grown without added phosphorus (as above), were 10.1 ± 1.0 and 1.5 ± 0.6 µg mg⁻¹ dry weight, respectively.

§ Within each column, values followed by different superscripts are significantly different (P < 0.05). Phytase activities were log₁₀-transformed prior to analysis.

15 Seedlings of the selected lines were grown in sterile nutrient solution and phytase activity was determined between days 10 and 12 of growth. Extracellular phytase activity was only detected in transgenic *A. thaliana* that contained gene constructs carrying the leader sequence. Under the assay conditions used, we determined that about 10% of the total soluble root phytase activity in these lines was secreted per
20 day as an extracellular enzyme into the growth medium (Table 6). In contrast,

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measurable phytase activity was not secreted from roots of the control plants carrying plasmid pBS389 alone, or from the roots of plants that expressed phytase without the leader sequence attached (Table 6). Accordingly, significant zones of phytate depletion were only observed around the roots of those transgenic lines containing the chimeric *ext::PhyA-1* or *ext::PhyA-2* genes (Figure 10).

TABLE 6
Extracellular phytase activity of transgenic *A. thaliana* (C24 ecotype) plants.

Transgenic plant line *		Secreted phytase activity (mU)		
		Activity per plant per day	Activity per gram f. wt per day	Activity per ug protein
Control	a	< 0.01 [†]	< 1.5	< 0.1
PhyA-2	a	< 0.01	< 1.5	< 0.1
Ext::PhyA-2	a	0.19 ± 0.04	26.0 ± 4.8	4.5 ± 0.9
PhyA-1	b	< 0.01	< 1.5	< 0.1
ext::PhyA-1	b	10.07 ± 1.04	1538.0 ± 171.5	79.1 ± 5.1

* The control line was transformed with pBS389.

[†] Each observation is the mean of 4 replicates and is shown ± 1 standard deviations. Where shown with <, the value was below the indicated limit for detection.

c) Plant Growth on phytate-containing media

The growth responses of transgenic plants in media supplemented with phytate were determined for transformed *A. thaliana* plants.

5 We compared the growth of transformed *A. thaliana* plants in media containing either no added phosphate, or using 0.8 mM Na₂HPO₄ or phytate (equivalent to 0.8 mM phosphate), as a source of phosphorus. Whilst plants transformed with the pBS389 control plasmid, or expressing the *PhyA-2* gene without the extensin leader sequence, could only grow efficiently in the presence of 0.8 mM Na₂HPO₄, plants
10 transformed with the gene construct pAER04 (Figure 6) and expressing the *extensin::PhyA-2* fusion protein grew efficiently on phytate as well soluble phosphate as a source of phosphorus. Accordingly, data shown in Figure 7 show that plants ectopically-expressing the extensin-phytase fusion polypeptide grow more rapidly on media containing phytate as a sole source of phosphorus.

15

We also compared the enhancement of growth, using phytate as a sole source of phosphorus, that was obtainable using the *PhyA-1* or *PhyA-2* genes. As shown in Figure 8, transformed *A. thaliana* plants carrying either the *PhyA-1* or *PhyA-2* genes fused to a nucleotide sequence encoding the carrot extensin leader sequence
20 grew efficiently in media having phytate as the sole source of phosphorus (see panels C, E of Figure 8). In contrast, plants expressing the phytase genes without the leader sequence (see panels B, D of Figure 8) attached grew poorly on phytate, as did plants not expressing any phytase gene (panel A of Figure 8). Accordingly, both modified phytase genes work equally-well to improve the phosphorus nutrition
25 of plants, however significantly more plant growth was obtained for plants expressing the extensin-phytase fusion polypeptide compared to plants expressing phytase but unable to target the phytase to the extracellular space.

To test the requirement for the extensin leader sequence, we also compared the
30 growth of plants expressing the *extensin::PhyA-1* fusion polypeptide on media lacking added phosphorus (Figure 9, panel A), to the growth of plants expressing the

PhyA-1 polypeptide without the extensin leader sequence on phytate (Figure 9, panel B), or plants expressing the PhyA-2 polypeptide on phytate (Figure 9, panel C), or expressing the extensin::PhyA-2 fusion polypeptide on phytate (Figure 9, panel D). After 40 days growth, at a density of about thirty plants per agar plate, significantly enhanced growth was observed on phytate, even for lines wherein the introduced phytase-encoding gene lacked nucleotide sequences encoding the carrot extensin leader sequence, compared to plants grown in the absence of a phosphorus source. However, optimum growth was clearly observed where the extensin leader sequence was present.

To demonstrate that the enhanced growth on phytate is due to enhanced utilisation of phytate, we also stained media plates with 0.03% (w/v) FeCl_3 to determine regions where phytate was absent from the medium after 22 days of growth. As shown in Figure 10, staining was observed along the margins of the roots in Panel C, showing the absence of phytate from the medium, which phytate has been utilised by plants expressing the extensin::PhyA-1 fusion protein and grown on phytate, but not soluble phosphorus (0.8 mM Na_2HPO_4). Accordingly, the enhanced nutrition on phytate-containing media is correlated with enhanced phytate utilisation by the transgenic plants.

EXAMPLE 2

Phytase activities of plant roots and localisation of activity

The phytase activities in the roots of a wide range of agriculturally important legume and grass species, including subterranean clover, burr medic, white clover, lucerne, tobacco, *A. thaliana*, wheat, phalaris, ryegrass and danthonia, have been determined.

In summary, phytase activities in extracts prepared from roots of these species ranged between 0.1 and 1.7 nkat g^{-1} root fresh wt (equivalent to 6.0 mU g^{-1} root fresh wt and 102.0 mU g^{-1} root fresh wt, respectively), or alternatively, 0.2 to 1.5 nkat mg^{-1} total protein (equivalent to 12.0 mU mg^{-1} protein and 90.0 mU mg^{-1}

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protein, respectively), with levels of activity increased by up to 3.3-fold (9.8-fold on a total root protein basis) when seedlings were grown in conditions of phosphorus deficiency.

5 In contrast, acid phosphatase activity measured in the same extracts ranged between 20 and 60 nkat g⁻¹ root fresh wt (equivalent to 1200 mU g⁻¹ root fresh wt and 3600 mU g⁻¹ root fresh wt, respectively). Phytase activity was a small component only (less than 5% for the range of species investigated) of the total acid phosphatase activity of plant roots, irrespective of the level of phosphorus nutrition.

10

The extracellular component of root phytase activity is a minor proportion of the total phytase activity measurable in the roots of naturally-occurring plants. For example, less than 0.042 nkat phytase activity g⁻¹ root fresh wt (ie., less than 4.7% of the activity measured in root extracts) could be eluted from roots of subterranean clover.

15 The estimated extracellular root phytase activity of intact roots of subterranean clover seedlings is as low as 0.03 nkat g⁻¹ root fresh wt (ie., 3% or less of the activity measured in soluble root extracts).

20 However, supplementation of subterranean clover roots with phytase, at a rate that is equivalent to 0.13 nkat g⁻¹ root fresh wt, resulted in a significant enhancement of the ability of the plants to acquire phosphorus from phytate.

EXAMPLE 3

Effect of organic acids on the phytase-labile component of soil phosphorus

25 We have also demonstrated that the efficacy of organic acids in improving the extractability of phosphorus from the soil, and to show that a significant component of the organic phosphorus in soil actually extracted by citrate is amenable to dephosphorylation by phytase.

30 Phytase-labile organic phosphorus was determined in various extracts prepared from two Australian pasture soils with contrasting fertiliser histories.

Materials and Methods

Soils

Soil samples were collected to a depth of 10 cm, under permanent pastures (containing perennial grass and annual legume components) located at two sites: (i) Rutherglen Research Institute, Victoria; and (ii) Ginninderra Experiment Station, Canberra, ACT. Individual cores (2.5 cm diameter) of soil (~30 samples) from each treatment at each site were bulked as a composite sample which was air-dried, passed through a 2 mm sieve to remove large, particulate matter and stored at room temperature.

Samples from the Rutherglen site were collected in 1993 from treatments of a non-replicated fertiliser trial, the details for which are published in Ridley *et al.* (1990). Briefly, the trial was established in 1914 on three 1.5 ha fields. Two fields (F_R and $F+L_R$) received approximately 125 kg ha^{-1} of single superphosphate (9% phosphorus) each alternate year from 1914 to 1986, while the third field (U_R) was unfertilised. One of the fertilised fields ($F+L_R$) was also top-dressed with lime from 1914 to 1948, receiving nine applications of 1.25 t ha^{-1} .

Samples from Ginninderra Experiment Station (Wallaroo 3 Paddock) were collected in October, 1996. Three phosphate fertiliser treatments were imposed across the trial on its establishment in 1994. Plots were either unfertilised (U_G) or received three autumn applications of triple superphosphate (20.7% phosphate), totalling 416 kg ha^{-1} ($F1_G$) or 675 kg ha^{-1} ($F2_G$).

Properties of the two soils are presented in Table 7. Total inorganic phosphorus and organic phosphorus were determined by the ignition-extraction ($0.5 \text{ M H}_2\text{SO}_4$) procedure (Olsen and Sommers 1982) and Colwell phosphorus by a 16 h extraction with 0.5 M NaHCO_3 (Colwell 1963), followed by determination of inorganic phosphorus using the molybdate-blue colour reaction (Murphy and Riley 1962). Organic carbon was determined by the Modified Mebius procedure (Nelson and Sommers 1982) and soil pH was measured in CaCl_2 .

Substrate specificities of phytase preparations

The substrate specificities of commercial preparations of wheat germ acid phosphatase (EC 3.1.3.2; Sigma Chemical Co.), *Aspergillus niger* phytase (EC 3.1.3.8; Sigma) and a purified preparation of the *A. niger* NRRL 3135 phytase (kindly provided by Dr Markus Wyss; F. Hoffmann-La Roche, Switzerland) were determined using a range of organic phosphorus compounds. The specific activities of the three enzyme preparations were tested at 27 °C, in 50 mM MES buffer (pH 5.5) containing 1 mM EDTA, against the following substrates: *myo*-inositol hexaphosphoric acid (dodecasodium salt; IHP), α -D-glucose 1-phosphate (disodium salt; G1P), ribonucleic acid (type VI; RNA), adenosine-5'-triphosphate (ATP), D(-)-3-phosphoglyceric acid (trisodium salt; PGA), *p*-nitrophenyl phosphate (disodium salt; *p*NPP), and *bis*(*p*-nitrophenyl) phosphate (sodium salt; *bis-p*NPP). With the exception of ATP (Boehringer Mannheim), all substrates were obtained from Sigma Chemical Co.

Assays were performed in 1 ml volumes, using 50 $\mu\text{g ml}^{-1}$ acid phosphatase; 1.14 $\mu\text{g ml}^{-1}$ commercial phytase; or 0.45 $\mu\text{g ml}^{-1}$ purified phytase. These amounts of enzyme were chosen on the basis of the reported specific activities of the preparations, against either IHP or *p*NPP. Enzyme assays were conducted over 30 min at a range of substrate concentrations between 2.4 mM and 4.8 mM phosphorus, with three replicates. Activities were measured against *p*-nitrophenol standards (Bessey et al. 1946) for the *p*NPP and *bis-p*NPP samples, and for all other samples by the release of inorganic phosphorus as determined using the malachite-green reaction (Irving and McLaughlin 1990).

Table 7
Properties of two pasture soils (0 to 10 cm depth) from Ginninderra and Rutherglen

Site	Soil Type (Stace et al. 1968)	Fertiliser treatment ^a	phosphorus (mg kg ⁻¹ soil; ignition/extraction method)		pH (CaCl ₂)	Organic C (%)	Colwell phosphorus (mg kg ⁻¹ soil)
			Total phosphorus	Inorganic phosphorus			
Ginninderra Experiment Station	Yellow podzolic	U _G (Unfertilised)	203	53	4.67	1.88	11.3
		F1 _G (86 kg P ha ⁻¹)	242	79	4.57	1.91	25.0
		F2 _G (140 kg P ha ⁻¹)	254	97	4.57	1.95	43.7
Rutherglen Research Institute	Grey-brown to yellow podzolics	U _R (Unfertilised)	154	30	4.60	1.99	7.8
		F _R (~450 kg P ha ⁻¹)	312	113	4.21	3.07	38.8
		F+L _R (~450 kg P ha ⁻¹ and 11.25 tonnes lime ha ⁻¹)	287	95	4.55	2.57	26.4

^a Total amount of fertiliser applied since inception of trial

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Measures of enzyme-labile soil

Soil extraction and total, organic and inorganic phosphorus determinations

Amounts of air-dried soil of between 2 and 10 g were extracted in 50 ml
5 polypropylene tubes using two volumes of sterile extractant solution, usually
deionised water, 50 mM (~1.0%) citric acid (pH ~2.3) or 0.5 M Na-bicarbonate (pH
8.5). Other extractants included 0.025 and 0.1 M HCl, and 0.01 M CaCl₂ (pH 5.5).
The soils were extracted at 22 ± 2 °C for 30 min on a reciprocal shaker (300 rpm),
followed by centrifugation (10, 300 g) for 15 min. Soil extracts were decanted from
10 the pelleted material and stored at 4 °C prior to enzyme analyses.

The phosphorus contents of soil extracts were determined on 1 ml sub-samples of
each solution. Inorganic phosphorus was determined by measuring the inorganic
phosphorus content of solutions with malachite-green reagent (Irving and
15 McLaughlin 1990). In order to determine total phosphorus, the samples were
autoclaved (120 kPa/ 121 °C; 40 min) in the presence of 0.6 M H₂SO₄ and 3.3%
ammonium persulphate (Schoenau and Huang 1991), and were similarly analysed
for inorganic phosphorus content. Corrections for volume loss during autoclaving
were made as necessary, based on gravimetric analyses. Organic phosphorus in
20 the extracts was calculated by deduction of inorganic from total phosphorus.

Incubation of soil extracts with enzyme

Standard assays involved incubation of 1 ml of soil extract in the presence of
excess enzyme: either 0.25 nkat (ie., 0.50 nkat g⁻¹ soil) of commercial phytase, as
25 determined against IHP substrate for the incubation conditions specified herein, or
1.14 nkat (2.28 nkat g⁻¹ soil) of purified phytase. These amounts of enzyme
equated to 1.56 (3.12 nkat g⁻¹ soil) and 0.03 nkat (0.05 nkat g⁻¹ soil) of acid
phosphatase activity as determined against pNPP, for the commercial and purified
preparations, respectively. Samples were adjusted to pH ~5.5 with either diluted
30 HCl or NaOH, 300 µl of 250 mM MES buffer (pH 5.5; containing 5 mM EDTA) was
added, and the solutions were made up to a final volume of 1.5 ml with deionised

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water. Immediately on addition of the enzyme to each reaction, an aliquot was removed and mixed with a one-fifth volume of 25% trichloro-acetic acid (TCA). Remaining solutions were incubated at 27 °C for 6 h, after which TCA was added to terminate the reaction. The TCA-treated samples were centrifuged in an Eppendorf
5 microfuge (12,000 rpm, 10 min) prior to analysis for inorganic phosphorus using malachite-green reagent. Control treatments were routinely included, whereby either soil extract or enzyme were omitted.

Statistics

10 Enzyme assays and other soil measures were replicated three times and the data were analysed to investigate variation associated with soil extraction and incubation procedures, using one- and two-way analyses of variance (ANOVAs). Where F-ratios were significant ($P < 0.05$), treatment means were compared by least significant difference (LSD).

15

Results

Substrate specificities and specific activities of phytase preparations

The specific activities of the three enzymes for IHP ranged from 1.0 nkat mg⁻¹ protein (wheat bran acid phosphatase) to 560.9 nkat mg⁻¹ (purified *A. niger* phytase;
20 Table 8). The purified *A. niger* phytase preparation had a 12-fold higher specific activity for IHP than the commercial phytase. Moreover, purified phytase showed a narrow substrate specificity, with specific activities for a range of organic phosphorus substrates that were 10% or less than the specific activity for IHP. The
25 commercial *A. niger* phytase preparation was less substrate-specific, with highest activity observed against pNPP. The activity profile of the commercial phytase preparation was similar to that of wheat bran acid phosphatase.

It is likely that the commercial *Aspergillus* phytase preparation contained a
30 considerable amount of phosphatase activity that was not specific for phytate. This was removed from the more purified preparation. The wheat bran acid

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phosphatase was not used in subsequent experiments because of its similarity to commercial phytase.

TABLE 8

5 **Substrate specificities of various phytase enzyme preparations**

Substrate	Specific Activity (nkat mg ⁻¹ protein)		
	Acid phosphatase	Sigma phytase	Purified phytase
<i>myo</i> -inositol hexakisphosphate	1.0	46.9	560.9
Glucose 1-phosphate	0.4	84.8	0.1
Ribonucleic acid	0.4	38.5	0.3
Adenosine triphosphate	4.2	55.2	59.4
Phosphoglyceric acid	3.6	78.7	54.5
<i>p</i> -nitrophenyl phosphate	7.0	293.0	13.1
<i>bis</i> (<i>p</i> -nitrophenyl) phosphate	0.2	11.5	35.5
LSD (<i>P</i> =0.05)	0.3	14.5	51.7

Inorganic and organic phosphorus contents of extracts from unfertilised Ginninderra soil

- 10 Unfertilised soil from Ginninderra Experiment Station (U_G) was selected initially for phosphorus measurements because of its low phosphorus status (Table 7). There was a requirement for soil extracts to contain sufficient levels of organic phosphorus, so that any change upon incubation with enzyme would be detectable. Various extracts of field-moist U_G soil were prepared, in which recoverable organic
- 15 phosphorus was determined. Water, 0.01 *M* CaCl₂, 0.025 *M* HCl and 0.1 *M* HCl extracted less than 1.0 µg organic phosphorus g⁻¹ soil. By contrast, up to 22.5 µg phosphorus g⁻¹ soil was extracted with citric acid and 0.5 *M* Na-bicarbonate. Na-

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bicarbonate, citric acid and water were thus selected as extractants for further analysis. A solution-to-soil ratio of 2:1 extracted higher concentrations of organic phosphorus (as compared to a 5:1 ratio) and was adopted for the subsequent incubation experiments.

5

Enzyme-labile phosphorus in extracts from fertilised Rutherglen soil.

Fertilised Rutherglen soil (F_R) was used to determine the appropriate conditions to be employed for enzyme incubation studies with all soil-extractant combinations. The hydrolysis of organic phosphorus from extracts of soil F_R was measured for up to 8 h in the presence of three concentrations of commercial or purified phytase, or in the absence of enzyme.

The release of inorganic phosphorus from incubated citric acid soil extracts is shown in Figure 14. Additions of 0.50 nkat commercial phytase or 2.28 nkat purified phytase g^{-1} soil were required for reactions to approach completion within 8 h. With lower amounts of enzyme, reactions proceeded more slowly and did not reach completion during the incubation period. For all subsequent measures of labile phosphorus, including various soils containing lower organic phosphorus concentrations, soil extracts were incubated for 6 h in the presence of 0.50 or 2.28 nkat phytase g^{-1} soil, using commercial or purified preparations, respectively.

To determine the contribution of soil microbe-derived enzyme activities to the observed rates of hydrolysis in amended soil extracts, duplicate samples were passed through 0.45 μm filters (Millex-HA, Millipore). There were no differences in either the rate or total quantity of inorganic phosphorus liberated from citric acid, water or Na-bicarbonate extracts of soil F_R , between filtered and unfiltered samples (data not shown). Therefore, over the 6 h incubation period, microbial enzyme activity did not contribute to the observed rates of hydrolysis. A low, and insignificant, level of hydrolysis was measured when no enzyme was added to the extracts (Figure 14).

Effects of citric acid concentration and pH on phytase-labile phosphorus from fertilised Rutherglen soil

Citric acid concentration

- 5 Extractable organic phosphorus from soil F_R increased significantly with increasing concentrations of citric acid to 50 mM ($P < 0.05$; Figure 15). The amounts of organic phosphorus that were hydrolysed by the commercial and purified phytases, and the proportion of the total organic phosphorus which was enzyme-labile, also increased with citric acid concentration. For the commercial phytase, labile phosphorus
- 10 represented 13.5% and 82% of the organic phosphorus in water and 50 mM citric acid soil extracts, respectively. Purified phytase-labile organic phosphorus was approximately half of that hydrolysed by the commercial enzyme preparation, representing between 1.8% and 44.7% of the total organic phosphorus across the same range of citric acid concentrations.

15

Effect of pH of citric acid

- Soil extractions were prepared using 50 mM citric acid solutions adjusted to between pH 2.3 and pH 6.0. Extractable organic phosphorus increased with pH, from 7.5 phosphorous g^{-1} soil at pH 2.3 to 25.3 μg phosphorus g^{-1} soil at pH 6.0
- 20 (Figure 16). However, enzyme-labile phosphorus was similar across the entire pH range, with $\sim 7.8 \mu g$ phosphorus g^{-1} soil and $\sim 4.5 \mu g$ phosphorus g^{-1} soil hydrolysed by commercial phytase and purified phytase, respectively (Figure 16).

Comparison between citric and hydrochloric acids as extractants

- 25 Total organic phosphorus, and commercial and purified phytase-labile organic phosphorus in water, citric acid and hydrochloric acid extracts from soil F_R are presented in Table 9.

TABLE 9

Extractable organic phosphorus (P_o), and enzyme-labile phosphorus ($\mu\text{g g}^{-1}$ soil) using two sources of phytase from *A. niger*, in water, citric acid and HCl extracts of the fertilised Rutherglen soil (F_R).
n.d. = not detectable.

Extractant	Organic P ($\mu\text{g g}^{-1}$ soil)	Enzyme-labile P_o ($\mu\text{g g}^{-1}$ soil)			
		Commercial phytase (% of P_o)		Purified phytase (% of P_o)	
Water	2.10	0.26	(12.4)	0.07	(3.3)
50 mM citric acid (pH 2.3)	7.65	6.15	(80.4)	3.35	(43.8)
50 mM HCl (pH 1.45)	1.01	0.09	(8.9)	n.d.	-
5 mM HCl, pH 2.3	2.09	0.17	(8.1)	0.07	(3.3)
LSD ($P=0.05$)	0.54	0.30		0.57	

- Two HCl extractants were used: one at the same molar concentration as citric acid (50 mM) and the other at the same pH (pH 2.3, at ~5 mM). When used at an equivalent molar concentration, HCl extracted only 13% of the quantity of organic phosphorus extracted with citric acid, and the enzyme-labile phosphorus component was negligible relative to that of citric acid extracts. When the concentration of HCl was adjusted for a solution of pH 2.3, the amount of extractable organic phosphorus was increased, but was still only 27% of that extracted by citric acid. Likewise, the enzyme-labile organic phosphorus component was less than 3% compared to citric acid. While commercial and purified phytase-labile organic phosphorus in 50 mM citric acid extracts represented 80% and 44% of the extractable organic

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phosphorus, respectively, less than 13% and 4% of organic phosphorus in the other extracts were hydrolysed by either of the enzyme preparations (Table 9).

5 Extractable and phytase-labile organic phosphorus from soils with different fertiliser histories

The total and organic phosphorus contents of water, 50 mM citric acid (pH ~2.3), and 0.5 M Na-bicarbonate (pH 8.5) extracts of soil from phosphorus fertiliser trials at two sites are illustrated in Figure 17, along with the fractions of extractable organic phosphorus that were hydrolysed by commercial and purified phytase during separate incubations.

While Na-bicarbonate extracted more organic phosphorus than the other solutions (between 12 and 30 $\mu\text{g g}^{-1}$ soil; Figure 17, panels e, f), citric acid extracted the most enzyme-labile organic phosphorus (up to 5.7 $\mu\text{g phosphorus g}^{-1}$ soil; Figure 17c, d), with between 56% and 79% of the organic phosphorus in citric acid extracts being hydrolysed by the commercial phytase preparation. In contrast, only 7 to 17% and 2 to 9% of the organic phosphorus in water and Na-bicarbonate extracts, respectively, was hydrolysed by the commercial phytase (Figure 17a, b; Figure 17e, f). A smaller component of the organic phosphorus was hydrolysed by purified phytase. In citric acid extracts, 28 to 40% of the organic phosphorus was purified phytase-labile (Figure 17c, d), while only 3 to 8% and 1 to 2% of the organic phosphorus in water and Na-bicarbonate extracts, respectively, was hydrolysed (Figure 17a, b; Figure 17e, f).

Organic, inorganic and enzyme-labile phosphorus extracted by citric acid was significantly higher ($P < 0.05$) in the fertilised Rutherglen soils (F_R and $F+L_R$) than the unfertilised soil (U_R ; Figure 17c). In addition, both total extractable organic phosphorus and the component which was phytase-labile, were greater for the fertilised soil (F_R) than soil which had received both fertiliser and lime ($F+L_R$). While organic and inorganic phosphorus extracted by Na-bicarbonate were significantly greater ($P < 0.05$) for both fertilised Rutherglen soils, only the treatment which had

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received fertiliser alone contained a higher level of extractable, commercial phytase-labile phosphorus than the unfertilised control (Figure 17e). No differences in fertiliser treatment were evident for purified phytase-labile phosphorus.

- 5 For the soil from Ginninderra, which had received only recent applications of phosphorus fertiliser, there were no differences in either extractable organic phosphorus or enzyme-labile phosphorus across fertiliser treatments (Figure 17). However, the extractable inorganic phosphorus component increased significantly ($P < 0.05$) with phosphorus fertility, in both citric acid and Na-bicarbonate extracts
10 (Figure 17d, f).

Discussion

Characterising phytase-labile organic phosphorus in extracts of soil

- Two *A. niger* phytase preparations with markedly different substrate specificities
15 were used to measure the amounts of enzyme-labile organic phosphorus present in extracts of soil. A commercial preparation of *A. niger* phytase (from Sigma Chemical Co.) showed activity against a range of organic phosphorus substrates, with highest activity for *p*NPP. In accordance with previous reports (Wyss et al. 1999), a purified form of the *A. niger* phytase was highly specific for phytate. The
20 contrasting substrate specificities of the two phytase preparations were exploited to characterise the enzyme lability of organic phosphorus extracted from soils. Material hydrolysed by the purified phytase was considered likely to be indicative of the phytate content of the extracts, whereas hydrolysis by the commercial "phytase" preparation reflected a more general acid phosphatase activity. However, it should
25 be noted that the activities of the two preparations were not entirely mutually exclusive, as shown by their activities against a range of organic phosphorus substrates (Table 8).

- In water extracts from soils collected from Ginninderra Experiment Station and
30 Rutherglen Research Institute, less than 8% of the organic phosphorus present was hydrolysed by the purified phytase and only 7 to 17% was hydrolysed by the

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commercial preparation (Figure 17). Previous measures of enzyme-labile organic phosphorus in water extracts of soils have not been made using phytase with a narrow specificity for IHP.

- 5 The present work suggests that low levels of phytate occur in soil solution (less than $0.22 \mu\text{g phosphorus g}^{-1} \text{ soil}$), and also that organic phosphorus esters accessible to a more general phosphatase (ie. commercial phytase) activity are present in only small quantities. In contrast, higher amounts of organic phosphorus in water extracts (between 42 and 70%; Pant et al. 1994) and soil solution (48 to 62%;
- 10 Shand and Smith 1997) from Scottish soils, were hydrolysed by a phytase (from wheat bran) which was not specific for phytate.

- Several extractants were used to assess enzyme-labile organic phosphorus. Sodium-bicarbonate has previously been considered to extract a component of soil
- 15 organic phosphorus which is readily mineralisable (Bowman and Cole, 1978). On this basis, sequential soil organic phosphorus extraction procedures have been developed to include bicarbonate-extractable organic phosphorus as a labile fraction (Hedley et al. 1982; Sharpley 1985). However, the present experiments do not support the supposition that bicarbonate-extractable organic phosphorus is
- 20 labile. While Na-bicarbonate extracted the largest quantities of organic phosphorus relative to other soil extractants (up to $30 \mu\text{g phosphorus g}^{-1} \text{ soil}$), only a small proportion (between 1 and 9%) was enzyme-labile, even when using phytase with activity against a wide range of phosphate esters (Figure 17). Otani and Ae (1999) have similarly shown that negligible amounts of organic phosphorus were enzyme-
- 25 labile in Na-bicarbonate extracts from a range of soils.

- In contrast, 50 mM citric acid extracted intermediate amounts of organic phosphorus ($3.8 \text{ to } 7.7 \mu\text{g phosphorus g}^{-1} \text{ soil}$), of which up to 79% could be hydrolysed by the commercial phytase preparation and up to 40% was hydrolysed by the purified
- 30 preparation. Otani and Ae (1999) also used a range of extractants and found that only citrate extracted organic phosphorus that was readily accessible to either acid

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phosphatase or broad-specificity phytase. From the present work, it is evident that citric acid and Na-bicarbonate extracts contain different components of the total organic phosphorus pool.

- 5 Up to 40% of citrate-extractable soil organic phosphorus was hydrolysed by the purified phytase preparation (Table 9; Figure 17), indicating that a considerable amount may occur as phytate. Citrate is an effective chelator of trivalent metal ions such as Fe^{3+} and Al^{3+} (Jones and Darrah 1994). In soils, citrate can release inorganic phosphorus into solution either by anion exchange with Fe- and Al-associated phosphates on soil adsorption surfaces (Gerke 1992), or by chelation of
10 precipitates to form soluble compounds (eg. Gardner et al. 1983). Phytate undergoes similar adsorption and precipitation reactions in soils to produce inorganic phosphorus (Ognalaga et al. 1994). It is conceivable that soil phytate is released into solution in the presence of citric acid via similar mechanisms.
- 15 Citrate also extracted organic phosphorus that was readily accessible to a phytase preparation possessing general acid phosphatase activity (Figure 17). The purified and commercial phytase preparations showed markedly different specific activities against a range of phosphate esters (Table 8). However, it was evident that they
20 also hydrolysed a common component of the citrate-extractable organic phosphorus; when combined, the amounts of extractable organic phosphorus hydrolysed by the two enzymes often exceeded the total quantity of extractable soil organic phosphorus (Figure 17). Enzyme-labile organic phosphorus in citric acid extracts may represent a component of soil phosphorus that can potentially be used
25 by plants. In previous work, we have observed that plants grown in sterile culture were able to use organic phosphorus substrates, such as glucose 1-phosphate and β -glycerophosphate, essentially as equivalent phosphorus sources to inorganic phosphorus for growth, while phytate was a relatively poor source of phosphorus (JE Hayes et al. submitted to *Plant and Soil*, AE Richardson, PA Hadobas and JE
30 Hayes submitted to *Plant, Cell and Environment*).

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Higher concentrations of citric acid extracted more soil organic phosphorus and also increased the proportion of organic phosphorus that was enzyme-labile. By contrast, HCl (used at the same molarity as citric acid) extracted minimal phytase-labile organic phosphorus. Consequently, the chelating properties of citric acid, rather than acidification effects, were considered to be largely responsible for the extraction of enzyme-labile organic phosphorus. The amount of extractable organic phosphorus also increased with the pH of the citric acid extractant. It is likely that this was due to the greater chelation ability of the citrate²⁻/citrate³⁻ species, which would predominate over citrate/citrate⁻ species at the higher pH. Gerke and Meyer (1995) found that citrate adsorption to a humic podzol was higher at pH 6.5 compared to pH 5.5. Concurrently, they observed greater mobilisation of phosphorus. In the present work, the amount of enzyme-labile organic phosphorus did not increase with higher citric acid pH.

Effect of fertiliser history on extractable, phytase-labile soil organic phosphorus

Measures of phosphorus in extracts of soils with contrasting fertiliser histories indicated that there were marked differences in the amounts of both total organic phosphorus and enzyme-labile organic phosphorus. Similar amounts of organic and hydrolysed phosphorus were observed in fertilised and unfertilised soils from plots at a recently established field site (Ginninderra). Only the inorganic phosphorus content of the soil at this site had been increased by the relatively recent phosphorus fertiliser applications. By contrast, clear distinctions could be made between fertiliser treatments for soil with a long history of fertiliser application, but which had received no fertiliser for seven seasons prior to sampling (Rutherglen). Differences between phosphorus treatments were especially evident in citric acid extracts, where the extracts from the fertilised soil contained approximately 3-fold greater amounts of enzyme-labile organic phosphorus than extracts from unfertilised soil. Extracts of soil which had received both fertiliser and lime similarly contained higher levels of total organic phosphorus and enzyme-labile organic phosphorus. These results may indicate that, with extended periods of

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fertiliser application, a greater proportion of applied phosphorus accumulates in soil as labile organic phosphorus. This component of labile organic phosphorus may make an important contribution to the phosphorus nutrition of pastures. Mineralisable fractions of soil organic phosphorus have been reported to be
5 important for phosphorus cycling within permanent pasture systems (McLaughlin et al. 1990).

Conclusions

We have shown that a only small component of the organic phosphorus in water
10 and Na-bicarbonate extracts of soil can be hydrolysed by phytase and general acid phosphatase activities. This may indicate that only low levels of potentially plant-available organic phosphorus occur in soil solution. By contrast, greater amounts of enzyme-labile organic phosphorus were extracted using citrate. Citrate is exuded into the rhizosphere by roots of a number of plant species and, in many instances,
15 exudation is enhanced under conditions of phosphorus deficiency (eg. Lipton et al. 1987; Hoffland et al. 1989; Grierson 1992; Keerthisinghe et al. 1998). Citrate exudation by plant roots is considered to be an important mechanism for increasing the acquisition of soil inorganic phosphorus. Our results imply that citrate also increases the availability to plants of soil organic phosphorus, by solubilising a
20 fraction that can be hydrolysed by enzymes.

Enzyme-labile soil organic phosphorus was also influenced by soil fertiliser history. While inorganic phosphorus was the dominant labile fraction in soils which had received recent applications of fertiliser, in soils of low fertility (eg. soil U_G) or with a
25 long history of fertiliser application (Rutherglen soil), the enzyme-labile component of citrate-extractable organic phosphorus was equivalent to, or exceeded the quantity of extractable inorganic phosphorus.

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WE CLAIM:

1. A method of enhancing the phosphorus nutrition of a plant comprising ectopically expressing in the root of a plant an isolated nucleic acid molecule encoding a phytase polypeptide for a time and under conditions sufficient for said phytase to be secreted from the root.
2. The method according to claim 1 wherein the secretion of phytase from the root is achieved by ectopically expressing the phytase as a fusion protein with a secretory signal peptide.
3. The method according to claim 2 wherein the secretory signal peptide is selected from the group consisting of the carrot extensin signal peptide and the lupin acid phosphatase signal peptide.
4. The method according to any one of claims 1 to 3 wherein the phytase polypeptide is from *Aspergillus niger*.
5. The method according to any one of claims 1 to 4 wherein the phytase polypeptide has at least about 93% identity to SEQ ID NO: 2.
6. The method according to claim 5 wherein the phytase polypeptide is selected from the group consisting of SEQ ID Nos: 2 and 4.
7. The method according to claim 5 wherein the phytase polypeptide is encoded by a nucleotide sequence selected from the group consisting of SEQ ID Nos: 1, 3 and degenerate nucleotide sequence thereto.
8. The method according to claim 5 wherein the phytase polypeptide is encoded by a nucleotide sequence contained within the plasmid assigned AGAL Accession No. NM99/06795

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9. A method of enhancing the phosphorus nutrition of a plant comprising ectopically expressing in the root of a plant an isolated nucleic acid molecule encoding a fusion polypeptide between a secretory signal peptide and a phytase polypeptide for a time and under conditions sufficient for said fusion polypeptide to be secreted from the root, wherein said isolated nucleic acid comprises a nucleotide sequence selected from the group consisting of SEQ ID Nos: 9, 11, the phytase-encoding nucleotide sequence contained in the plasmid assigned AGAL Accession No. NM99/06795, and degenerate nucleotide sequences thereto.
10. The method according to claim 9 wherein the fusion polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID Nos: 10 and 12.
11. A method comprising: (i) ectopically expressing in the root of a plant an isolated nucleic acid molecule encoding a phytase polypeptide for a time and under conditions sufficient for said phytase to be secreted from the root; and (ii) modifying the chemistry of the soil around the root or other growth medium around the root using an organic acid.
12. The method according to claim 11 wherein the organic acid is citric acid.
13. The method according to claims 11 or 12 wherein the secretion of phytase from the root is achieved by ectopically expressing the phytase as a fusion protein with a secretory signal peptide.
14. The method according to claim 13 wherein the secretory signal peptide is selected from the group consisting of the carrot extensin signal peptide and the lupin acid phosphatase signal peptide.

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15. The method according to any one of claims 11 to 14 wherein the phytase polypeptide is from *Aspergillus niger*.
16. The method according to any one of claims 11 to 15 wherein the phytase polypeptide has at least about 93% identity to SEQ ID NO: 2.
17. The method according to claim 16 wherein the phytase polypeptide is selected from the group consisting of SEQ ID Nos: 2 and 4.
18. The method according to claim 17 wherein the phytase polypeptide is encoded by a nucleotide sequence selected from the group consisting of SEQ ID Nos: 1, 3 and degenerate nucleotide sequence thereto.
19. The method according to claim 17 wherein the phytase polypeptide is encoded by a nucleotide sequence contained within the plasmid assigned AGAL Accession No. NM99/06795.
20. A method comprising: (i) ectopically expressing in the root of a plant an isolated nucleic acid molecule encoding a fusion polypeptide between a secretory signal peptide and a phytase polypeptide for a time and under conditions sufficient for said fusion polypeptide to be secreted from the root, wherein said isolated nucleic acid comprises a nucleotide sequence selected from the group consisting of SEQ ID Nos: 9, 11, a phytase-encoding nucleotide sequence contained within the plasmid assigned AGAL Accession No. NM99/06795, and degenerate nucleotide sequences thereto and (ii) modifying the chemistry of the soil around the root or other growth medium around the root using an organic acid.
21. The method according to claim 20 wherein the organic acid is citric acid.

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22. The method according to claims 20 or 21 wherein the fusion polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID Nos: 10 and 12.
23. The method according to any one of claims 11 to 22 when used to enhance the phosphorus nutrition of a plant or the growth of a plant on a phosphorus source comprising phytate and/or increase the phosphorus content of a plant.
24. The method according to any one of claims 11 to 22 when used to enhance the biomass produced by a plant .
25. The method according to any one of claims 11 to 22 when used to enhance the rate of hypocotyl production or the rate of epicotyl production.
26. A transformed plant that ectopically expresses a secretable phytase polypeptide in its roots wherein said phytase polypeptide is secreted from the roots of said plant, wherein said plant is produced by a process comprising performing the method according to any one of claims 1 to 25.
27. Progeny of the transformed plant of claim 26 wherein said progeny ectopically expresses a secretable phytase polypeptide in its roots wherein said phytase polypeptide is secreted from the roots of said plant.
28. The transformed plant of claim 26 wherein said plant grows on a phosphorus source comprising phytate more efficiently than an isogenic plant that does not ectopically express the phytase enzyme.
29. The progeny of claim 27 wherein said progeny grows on a phosphorus source comprising phytate more efficiently than an isogenic plant that does not ectopically express the phytase enzyme.

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30. The transformed plant of claims 26 or 28 wherein said plant exhibits a larger biomass than an isogenic plant that does not ectopically express the phytase enzyme when grown on a phosphorus source comprising phytate.
- 5 31. The progeny of claims 27 or 29 wherein said progeny exhibits a larger biomass than an isogenic plant that does not ectopically express the phytase enzyme when grown on a phosphorus source comprising phytate.
- 10 32. The transformed plant of claims 26 or 28 wherein said plant exhibits an enhanced rate of epicotyl or hypocotyl production than an isogenic plant that does not ectopically express the phytase enzyme when grown on a phosphorus source comprising phytate.
- 15 33. The progeny of claims 27 or 29 wherein said progeny exhibits an enhanced rate of epicotyl or hypocotyl production than an isogenic plant that does not ectopically express the phytase enzyme when grown on a phosphorus source comprising phytate.
- 20 34. A process comprising producing a plant that ectopically expresses phytase in secretable form in its roots or a progeny of said plant that expresses said phytase in secretable form and growing said plant or progeny in a plant growth medium comprising phytate and a suitable carrier for application to plants and/or the growth medium.
- 25 35. The process according to claim 34 further comprising modifying the chemistry around the root of the plant or progeny using an organic acid.
36. The process according to claim 35 wherein the organic acid is citric acid.

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19 01.01

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37. The process according to any one of claims 34 to 36 wherein the secretion of phytase from the root is achieved by ectopically expressing the phytase as a fusion protein with a secretory signal peptide.
38. The process according to claim 37 wherein the secretory signal peptide is selected from the group consisting of the carrot extension signal peptide and the lupin acid phosphatase signal peptide.
39. The process according to any one of claims 34 to 38 wherein the phytase polypeptide is from *Aspergillus niger*.
40. The process according to any one of claims 34 to 39 wherein the phytase polypeptide has at least about 93% identity to SEQ ID NO: 2.
41. The process according to claim 40 wherein the phytase polypeptide is selected from the group consisting of SEQ ID Nos: 2 and 4.
42. The process according to claim 40 wherein the phytase polypeptide is encoded by a nucleotide sequence selected from the group consisting of SEQ ID Nos: 1, 3 and degenerate nucleotide sequences thereto.
43. The process according to claim 5 wherein the phytase polypeptide is encoded by a nucleotide sequence contained within the plasmid assigned AGAL Accession No. NM99/06795.
44. An isolated nucleic acid molecule encoding a mature phytase polypeptide without a phytase leader sequence and comprising a nucleotide sequence selected from the group consisting of: (i) the nucleotide sequence of SEQ ID NO: 1 or 9; (ii) a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 2 or 10; and (iii) a sequence that hybridises to a phytase-encoding nucleotide sequence contained within the plasmid assigned AGAL

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Accession No. NM99/06795 or a complementary nucleotide sequence thereto under high stringency hybridisation conditions.

45. The isolated nucleic acid molecule of claim 44 comprising the nucleotide sequence set forth in SEQ ID NO: 1 or 9.
46. A gene construct comprising the isolated nucleic acid molecule according to any one of claims 44 or 45 placed operably in connection with a promoter sequence that is operable in the root cells of a plant.
47. The gene construct of claim 46 comprising the *PhyA-2* chimeric gene sequence set forth in SEQ ID NO: 1.
48. The gene construct of claim 46 comprising the *ext::PhyA-2* sequence set forth in SEQ ID NO: 9.
49. The gene construct according to claim 46 consisting of the plasmid assigned AGAL Accession No. NM99/06795 .

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(54) Title: EXPRESSION OF PHYTASE IN PLANTS AS A METHOD OF MODIFYING PLANT PRODUCTIVITY

(57) Abstract: The present invention provides a method of modifying plant productivity comprising expressing in a plant cell, tissue or organ one or more genes capable of facilitating a plant's ability to utilise soil phosphorus, and, in particular, a phytase gene, wherein said phytase gene expresses a phytase polypeptide in secretable form. More particularly, the present invention provides a method of increasing plant productivity comprising expressing in the root of a plant an isolated nucleic acid molecule encoding a phytase polypeptide for a time and under conditions sufficient for said phytase to be secreted from the root. In a preferred embodiment of the invention the chemistry of the soil around the root is modified by the application of an organic acid. The invention also provides novel phytase-encoding genes; genetic constructs which are useful for performing the inventive method; and transgenic plants produced therewith having improved productivity compared to their otherwise isogenic counterparts.

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FIGURE 1a
FIGURE 1b
FIGURE 1c
FIGURE 1d
FIGURE 1e

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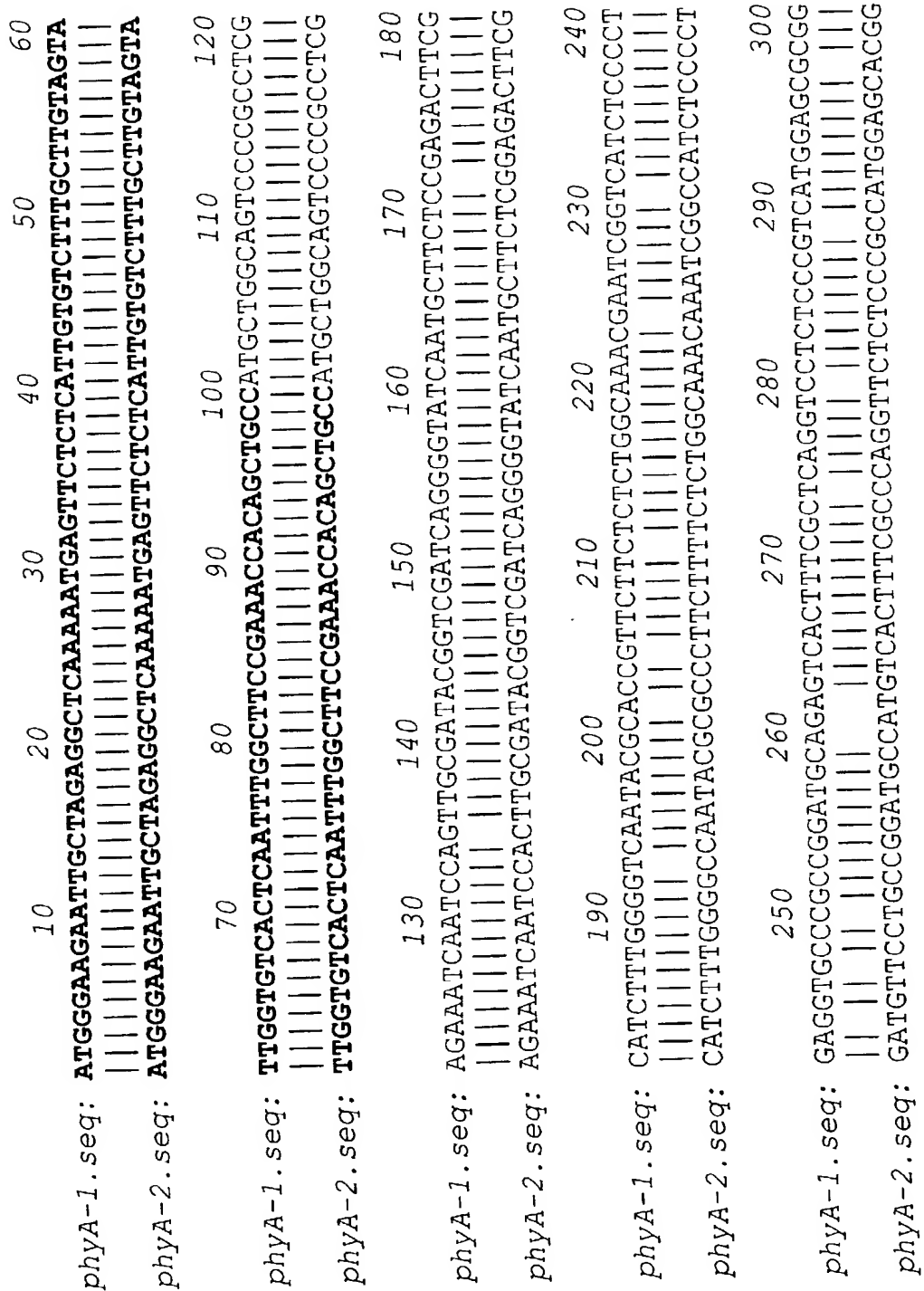


FIGURE 1a

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310 320 330 340 350 360
phyA-1. seq: TATCCGACCGACTCCAAGGCAAGAAATACTCCGCTCTCATTTGAGGAGATCCAGCAGAAC
|||||
phyA-2. seq: TATCCGACCGACTCCAAGGCAAGAAATACTCCGCTCTCATTCGAGGAGATCCAGCAGAAC
|||||

370 380 390 400 410 420
phyA-1. seq: GCGACCACCTTTGACGGAAATAATGCGCTTCCTGAAGACATACAACATACAGCTTGGGTGCA
|||||
phyA-2. seq: GCGACAACCTTCGAGGGAAATAATGCGCTTCCTGAAGACATACAACATACAGCCTGGGCGCG
|||||

430 440 450 460 470 480
phyA-1. seq: GATGACCTGACTCCCTTCGGAGAACAGGAGCTAGTCAACTCCGGCATCAAGTTCTACCAG
|||||
phyA-2. seq: GATGATCTGACTCCCTTCGGAGAGCAGGAGCTGGTCAACTCCGGCGTCAAGTTCTACCAG
|||||

490 500 510 520 530 540
phyA-1. seq: CGGTACGAATCGCTCACAAAGGAACATCGTTCCATTCAATCCGATCCTCTGGCTCCAGCCGC
||
phyA-2. seq: CGATACGAATCGCTCACAAAGAAACATTGTCCCGTTCAATCCGATCCTCAGGCTCCAACCGC
||

550 560 570 580 590 600
phyA-1. seq: GTGATCGCCTCCGGCAAGAAATTCAATCGAGGGCTTCCAGAGCACCAGCTGAAGGATCCT
|||||
phyA-2. seq: GTGATTGCCTCTGGCAATAAATTCAATCGAGGGCTTCCAGAGCAGCTAAGCTGAAGGATCCT
|||||

FIGURE 1b

610 620 630 640 650 660
phyA-1. seq: CGTGCCAGCCCCGGCCAAATCGTCGCCCAAGATCGACGTGGTCATTTCCGAGGCCAGCTCA
|||||
phyA-2. seq: CGTGCCAGCCCCGGCCAAATCGTCGCCCAAGATCGACGTGGTCATTTTCAGAGGCCAGCAC
670 680 690 700 710 720
phyA-1. seq: TCCAACAACACTCTCGACCCAGGCACTGCACTGTCTTCGAAGACAGCGAATTGGCCGAT
|||||
phyA-2. seq: TCCAACAACACTCTCGATCCGGGCACCTGCACCGTTTTCGAAGATAGCGAATTGGCCGAT
730 740 750 760 770 780
phyA-1. seq: ACCGTCGAAGCCAAATTTCACCGCCACGTTTCGTCCCCCTCCATTCGTCAACGCTGGAGAAC
|
phyA-2. seq: GACATCGAAGCCAAATTTCACCGCCACGTTTCGTCCCCCTCCATTCGTCAACGCTGGAGAAAT
790 800 810 820 830 840
phyA-1. seq: GACCTGTCCGGTGTGACTCTCACAGACACAGAAGTGACCTACCTCATGGACATGTGCTCC
|||
phyA-2. seq: GACTTGTCTGGCGTGTCTCTCACGGACACAGAAGTGACCTACCTCATGGACATGTGCTCC
850 860 870 880 890 900
phyA-1. seq: TTCGACACCATCTCCACCAGCACCCGTCGACACCAAGCTGTCCCCCTTCTGTGACCTGTTC
|||||
phyA-2. seq: TTCGACACCATCTCCACCAGCACCCGTCGACACCAAGCTGTCCCCCTTCTGTGACCTGTTC

FIGURE 1c

FIGURE 1d

phyA-1. seq: GTGGAGAATATCACCACAGACATGGATTCTCGTCTGCTTGGACGGTTCGGTTTGGCTTCTGCTTCCG 1210 1220 1230 1240 1250 1260

phyA-2. seq: GCGGAGAATATCACCACAGACCGATGGGTTCTCATCTGCCTGGACGGTTCCTTTCCGCGTCCG 1270 1280 1290 1300 1310 1320

phyA-1. seq: CGTTGTACGTCGAGATGATGCAGTGTCAAGCGGAGCAGGACCGCTGGTCCGTGTCCTTG 1330 1340 1350 1360 1370 1380

phyA-2. seq: CGCATGTACGTCGAGATGATGCAATGCCAGTCCGAGCAGGAGCCCTTGGTCCGTGTCCTTG 1390 1400 1410 1420 1430 1440

phyA-1. seq: GTTAATGATCGCGTTGTCCCGCTGCATGGGTGTCCGGTTGATGCTTTGGGAGAGATGTACC 1449

phyA-2. seq: GTTAATGATCGTGTGTTCGGCTGCATGGCTGTCCGGTTGATGCTTTGGGAAGATGTACG 1450

phyA-1. seq: CCGGATAGCTTTGTGAGGGGTTGAGCTTTGCTAGATCTGGGGGTGATTGGGCCGGAGTGT 1451

phyA-2. seq: CCGGATAGCTTCGTGAAGGGTTGAGCTTTGCCAGATCTGCCGGTGATTGGGCCGGAGTGT 1452

1449

phyA-1. seq: TTTGCTTAG

phyA-2. seq: TTTGCTTAG

FIGURE 1e

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FIGURE 2a

FIGURE 2b

phyA-1.pro: MGRIARGSKMSSLLVSLVLSNLASETTAAMLAVPASRNQSSCDTVD
|||||
phyA-2.pro: MGRIARGSKMSSLLVSLVLSNLASETTAAMLAVPASRNQSTCDTVD
|||||
phyA-1.pro: QGYQCFSETSHLWGQYAPFFSLANESVISPEVPAGCRVTFAQVLSRHGAR
|||||
phyA-2.pro: QGYQCFSETSHLWGQYAPFFSLANKSAISPDVPAGCHVTFAQVLSRHGAR
|||||
phyA-1.pro: YPTDSKGKKYSALIEEIQQNATTFDGKYAFLKTYNYSLGADDLTPFGEQE
|||||
phyA-2.pro: YPTDSKGKKYSALIEEIQQNATTFEGKYAFLKTYNYSLGADDLTPFGEQE
|||||
phyA-1.pro: LVNSGIKFYQRYESLTRNIVPFIRSSGSSRVIASGKKFIEGFQSTKLKDP
|||||
phyA-2.pro: LVNSGVKFYQRYESLTRNIVPFIRSSGNSNRVIAAGNKFIEGFQSTKLKDP
|||||
phyA-1.pro: RAQPGQSSPKIDVVISSEASSNNTLDPGTCTVFEDSELADTVEANFTATF
|||||
phyA-2.pro: RAQPGQSSPKIDVVISSEASTSNNTLDPGTCTVFEDSELADTVEANFTATF
|||||

FIGURE 2a

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260 270 280 290 300
phyA-1.pro: VPSIRQRLNDLSGVTLTDTTEVTYLMDCSFDTISTSTVDTKLSPFCDLF
|||||
phyA-2.pro: VPSIRQRLNDLSGVS LTDTTEVTYLMDCSFDTISTSTVDTKLSPFCDLF
310 320 330 340 350
phyA-1.pro: THDEWINYDY LQSLKKYYGHGAGNPLGPTQGVGYANELIARLTHSPVHDD
|| |||||
phyA-2.pro: THEEWINYDY LQSPNKYYGHGAGNPLGPTQGVGYANELIARLTHSPVHDD
360 370 380 390 400
phyA-1.pro: TSSNHTLDSSPATEFPLNSTLYADFSDNGIISILFALGLYNGTKPLSSTT
|||||
phyA-2.pro: TSSNHTLDSPATEFPLNSTLYADFSDNGIISILFALGLYNGTKPLSSTT
410 420 430 440 450
phyA-1.pro: VENITQTDGFSSAWTVPFASRLYVEMMQCQAEQEP LVRVLVNDRVVPLHG
|||||
phyA-2.pro: AENITQTDGFSSAWTVPFASRMVYVEMMQCQSEQEP LVRVLVNDRVVPLHG
460 470 480
phyA-1.pro: CPVDALGRCTRDSFVRGLSFARSGGDWAECEFA
|||||
phyA-2.pro: CPVDALGRCTRDSFVKGLSFARSGGDWAECEFA

FIGURE 2b

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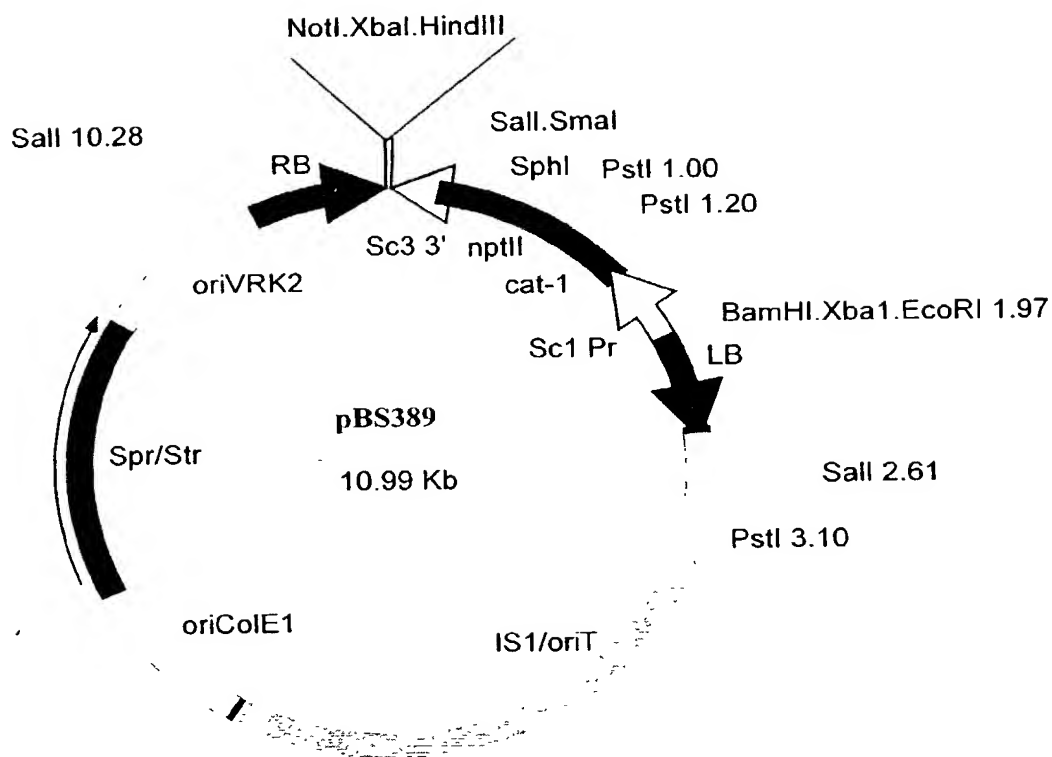


FIGURE 3

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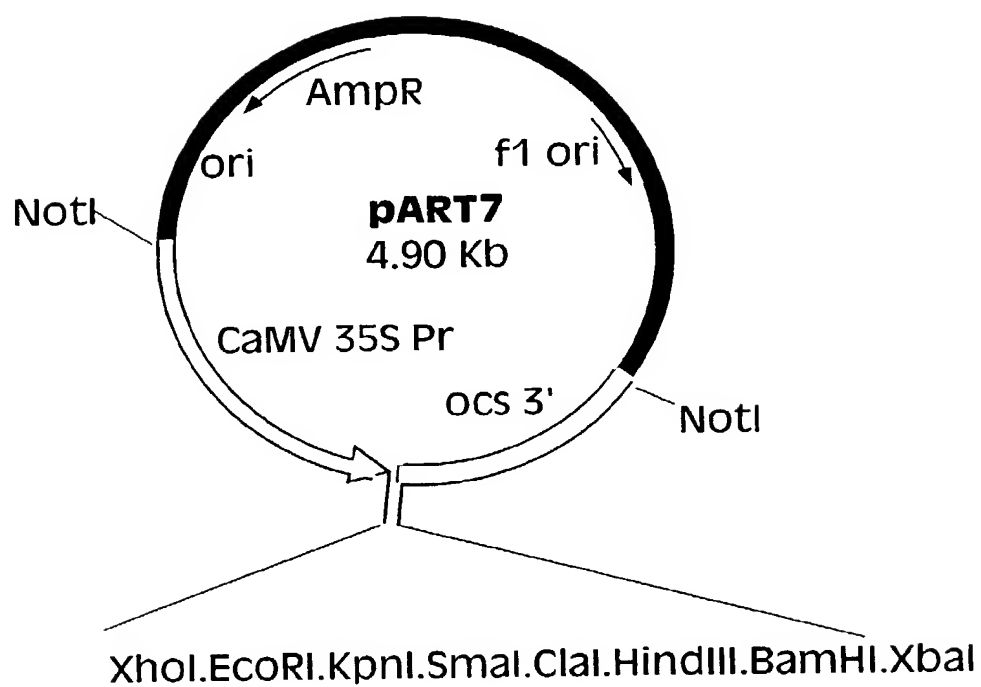


FIGURE 4

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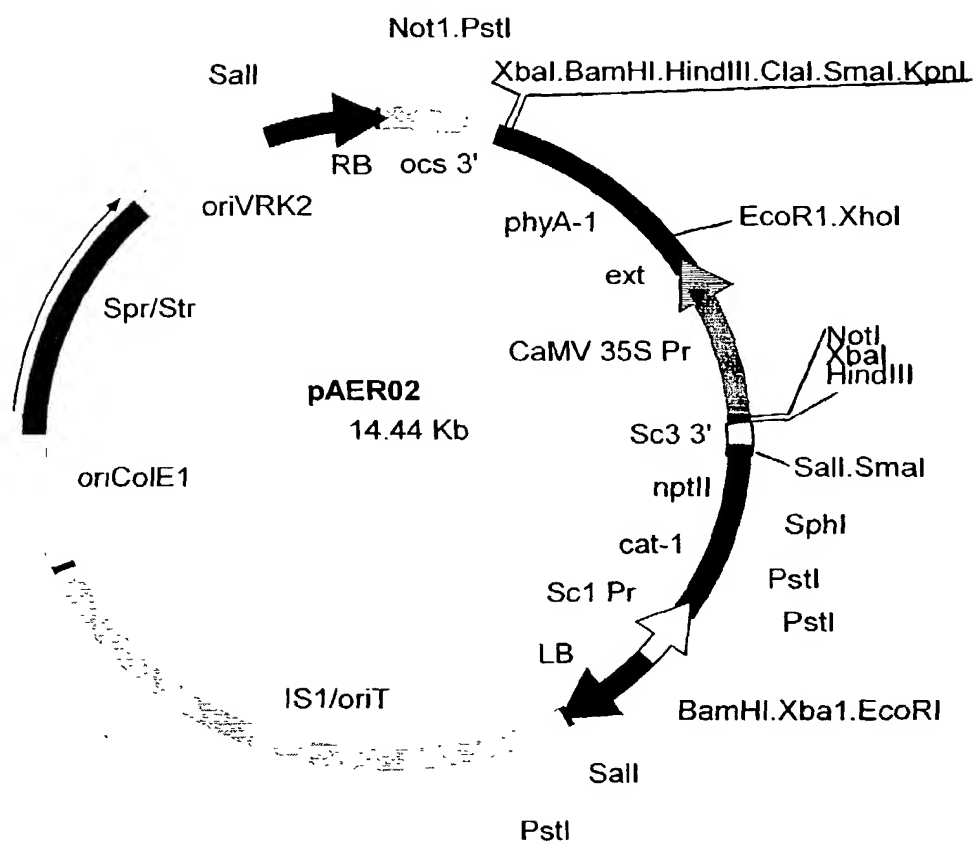


FIGURE 5

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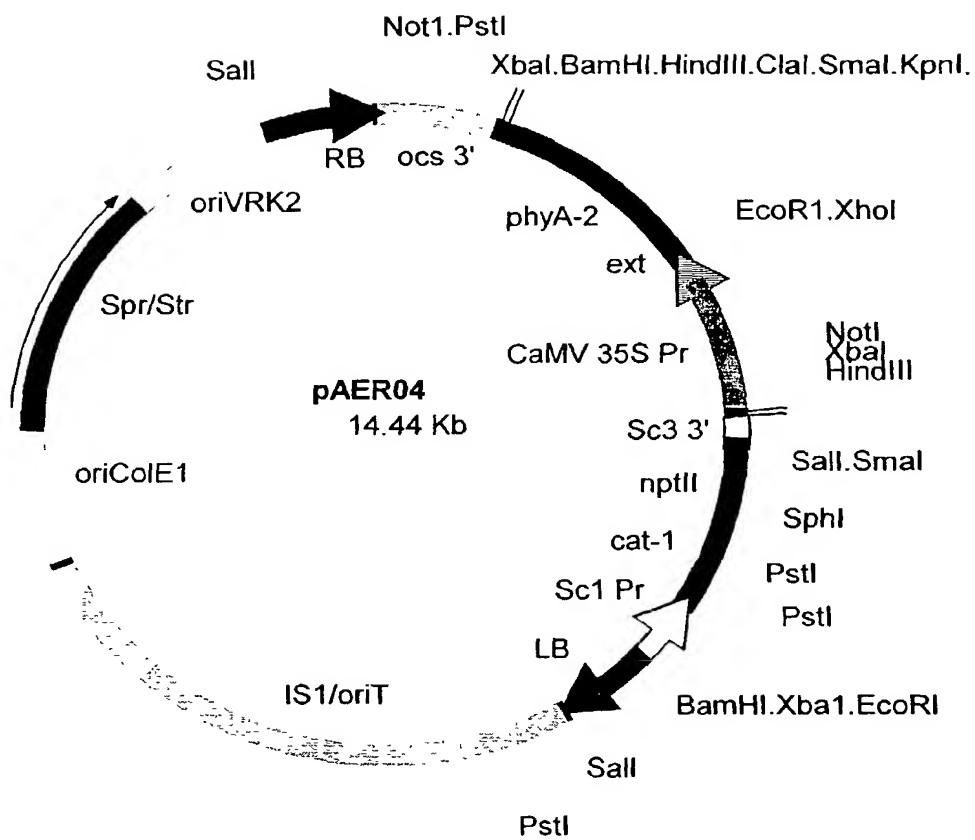


FIGURE 6

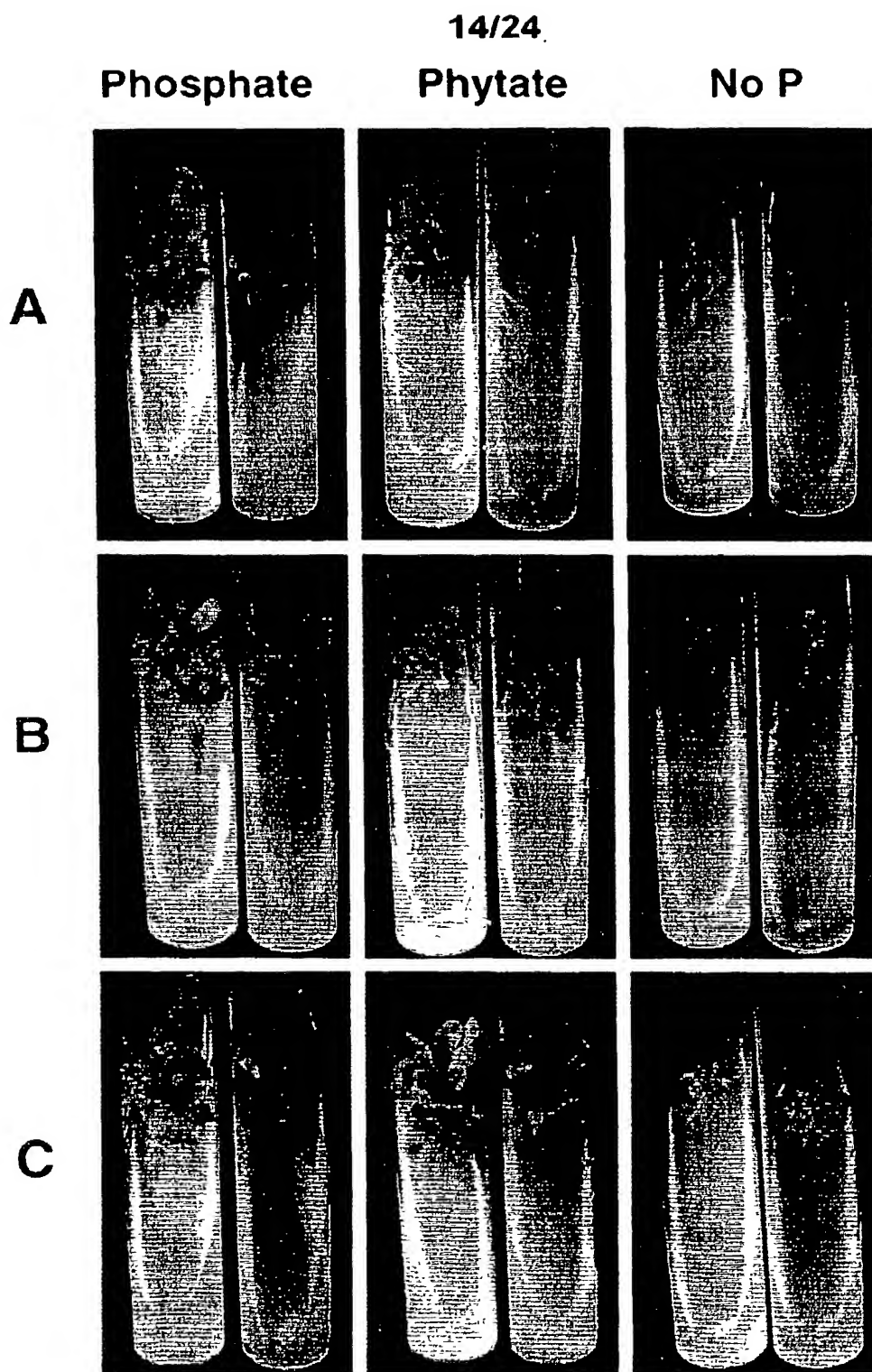


FIGURE 7

Plants supplied with phytate

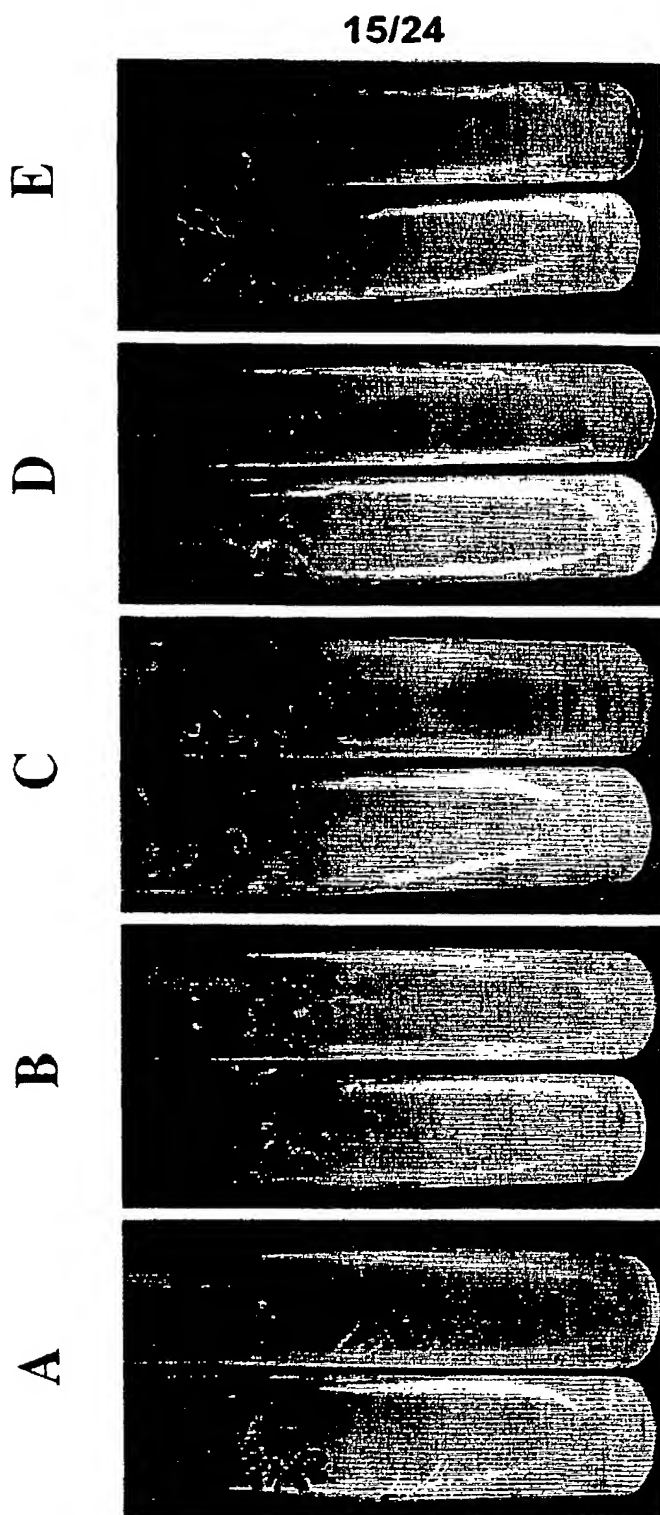


FIGURE 8

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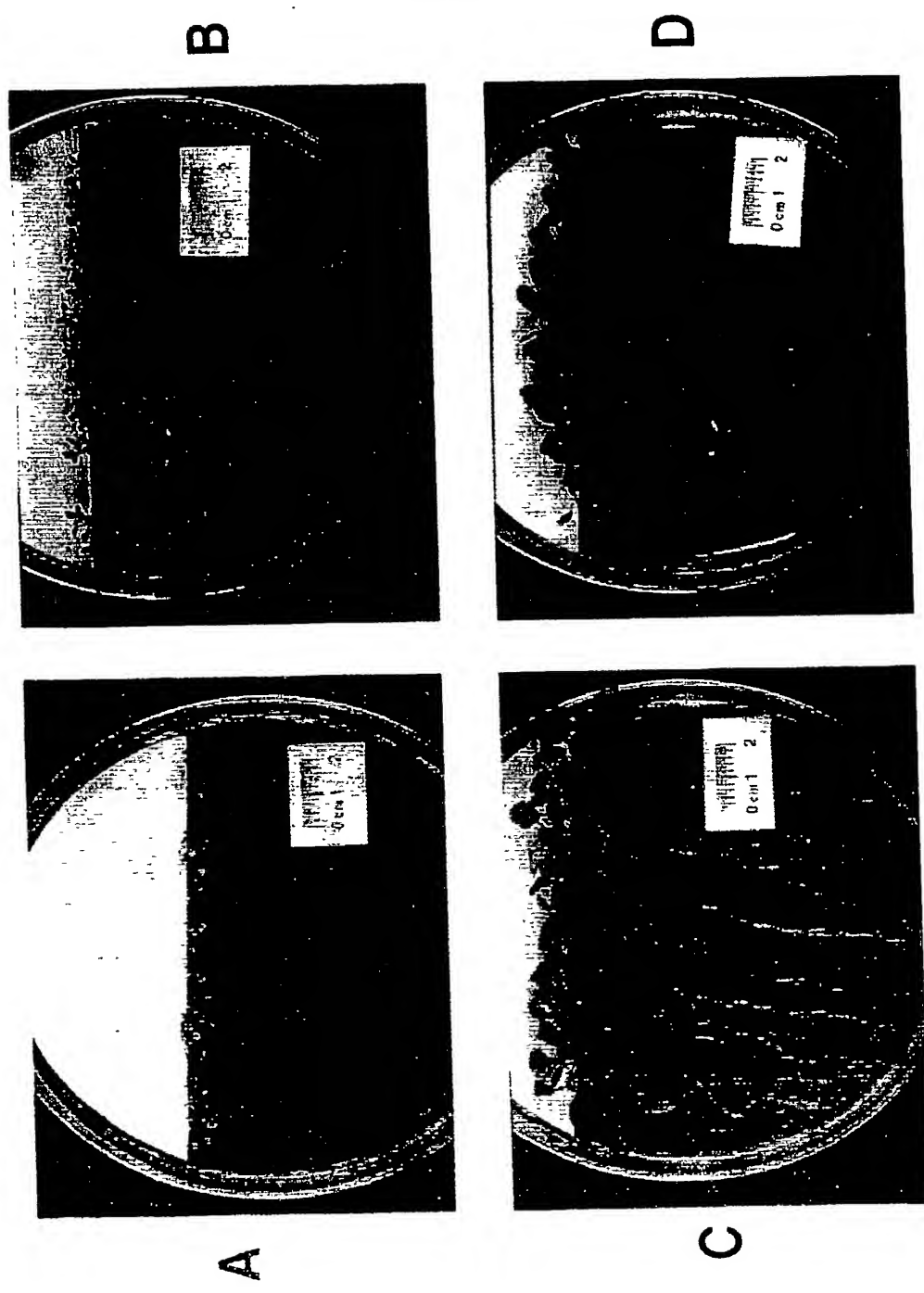
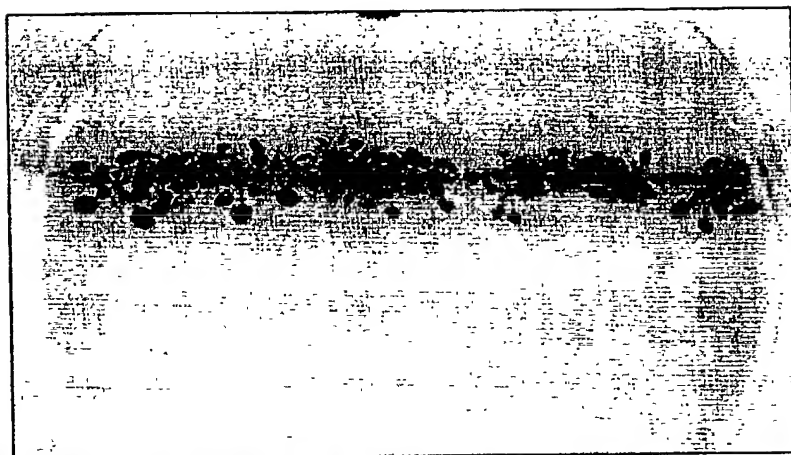


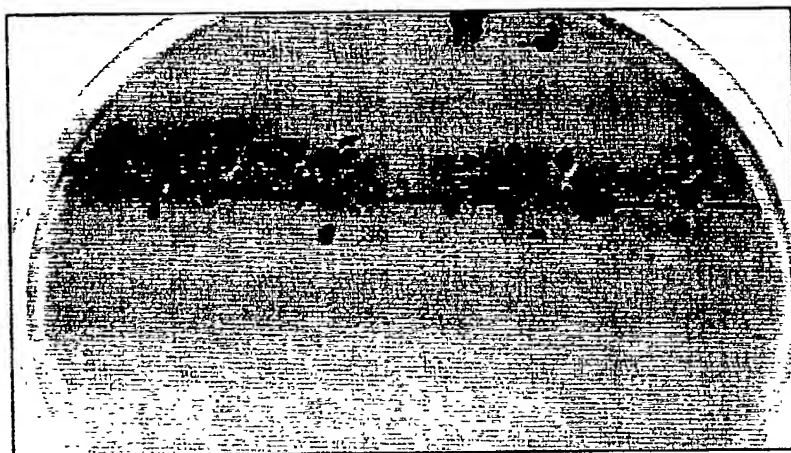
FIGURE 9

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A



B



C

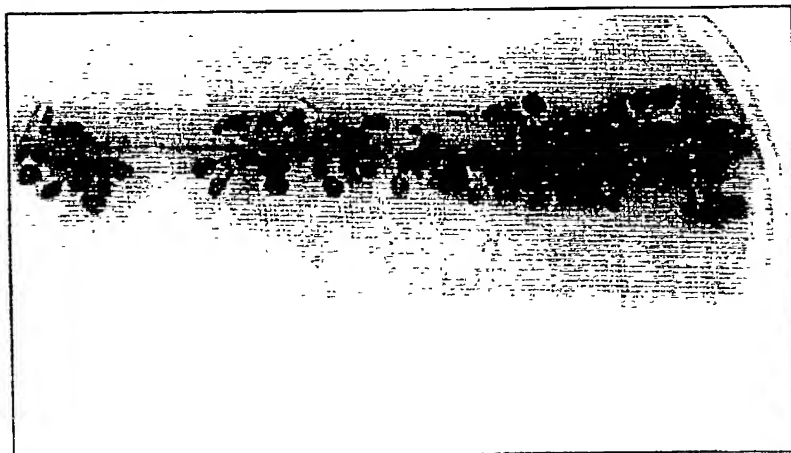


FIGURE 10

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Transgenic *Arabidopsis*

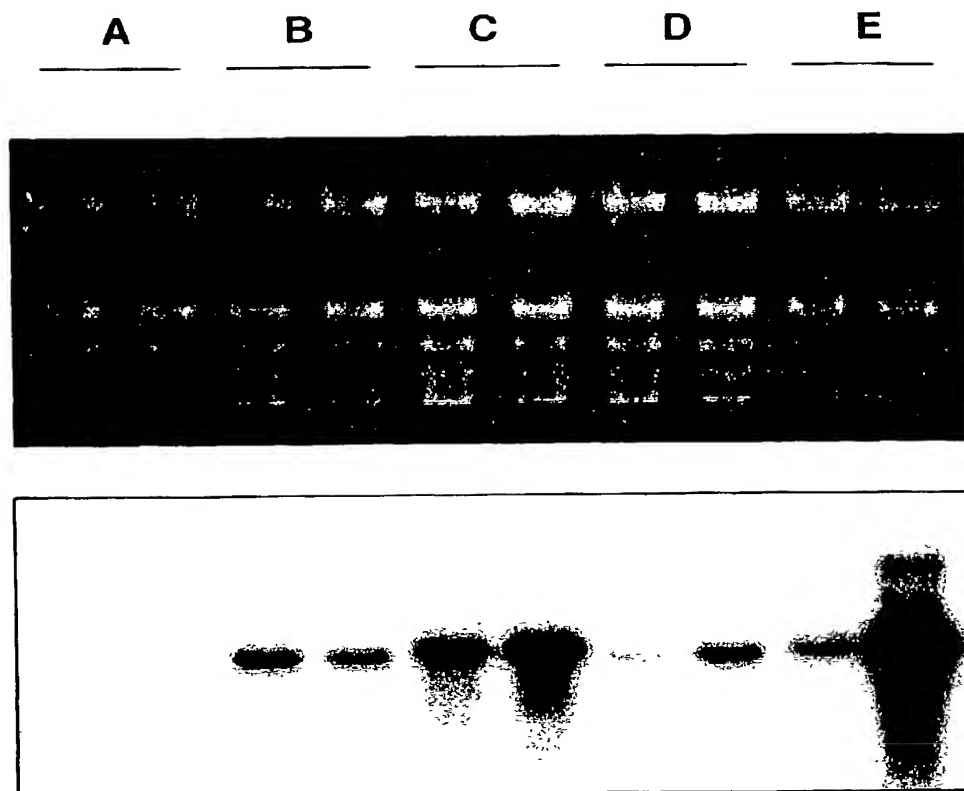
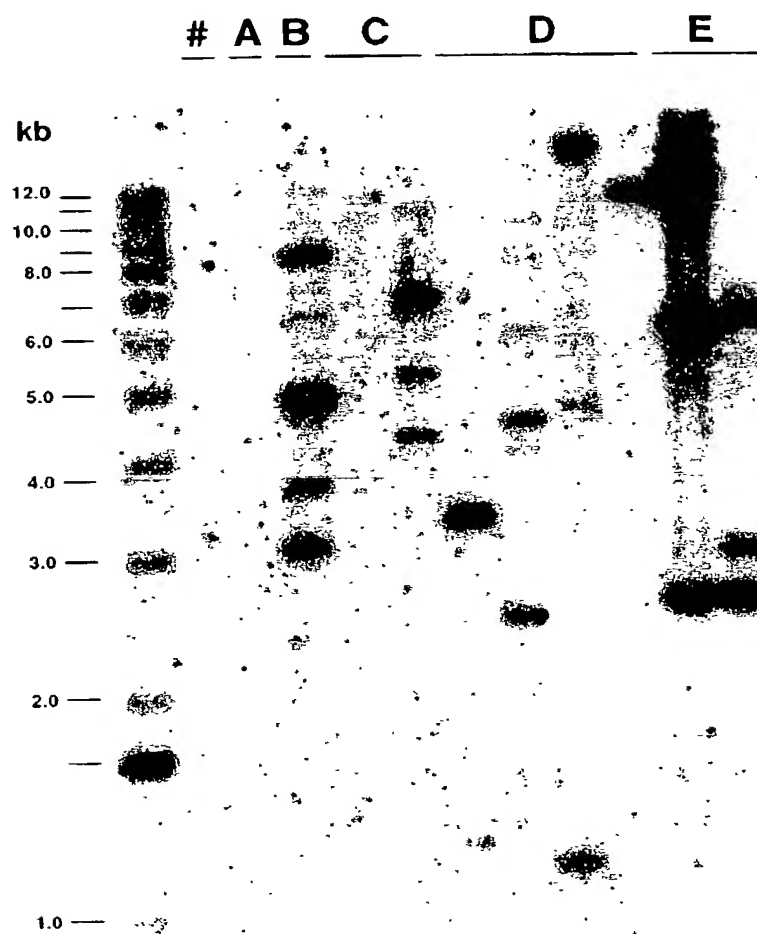


FIGURE 11

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Transgenic *Arabidopsis***FIGURE 12**

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Transgenic subterranean clover

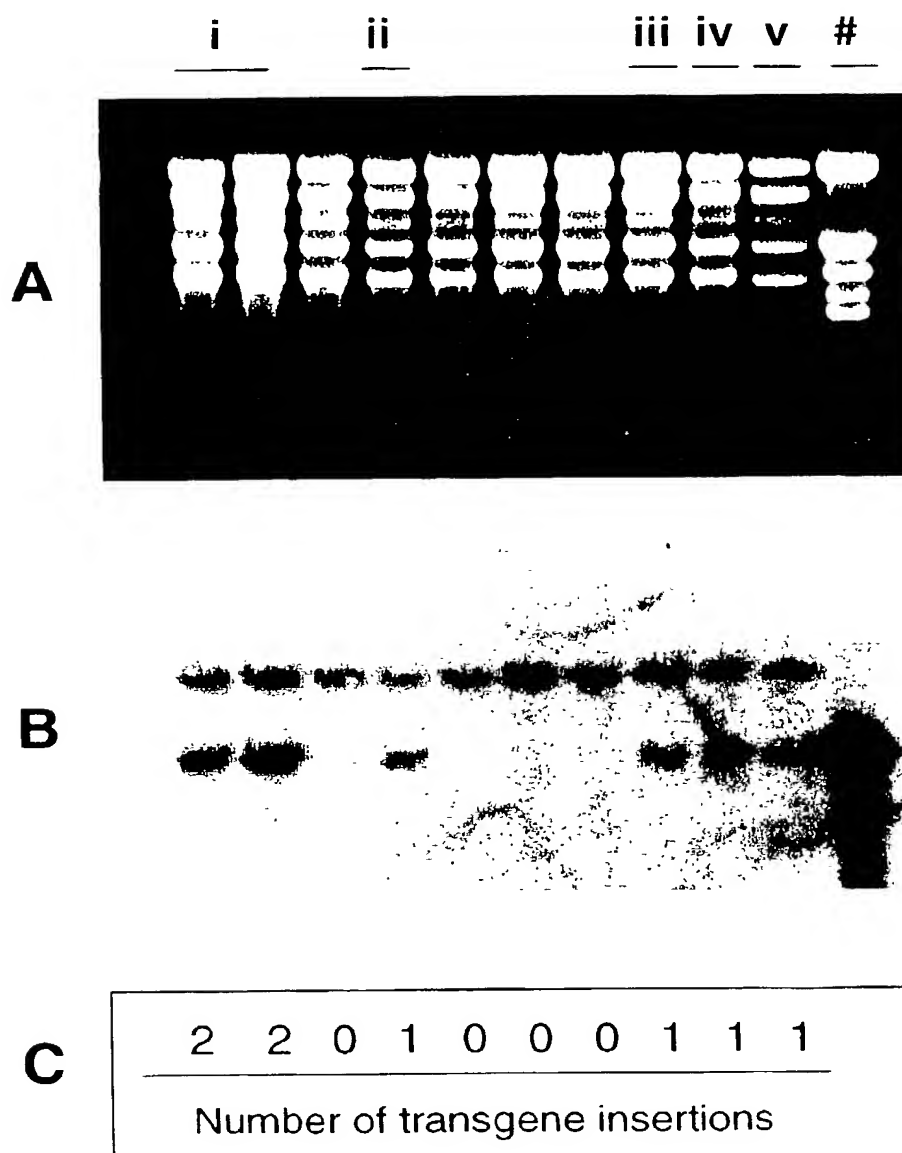


FIGURE 13

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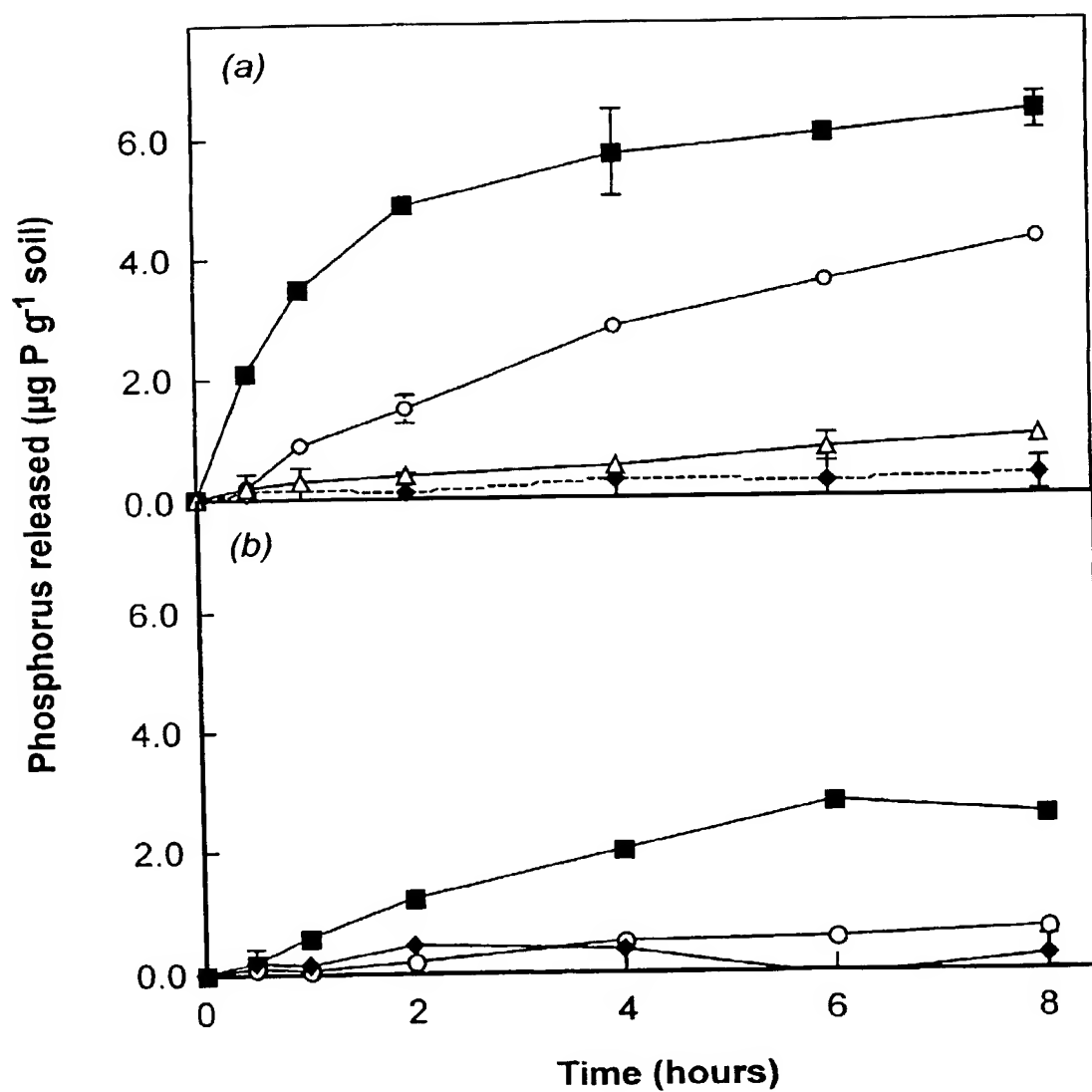


FIGURE 14

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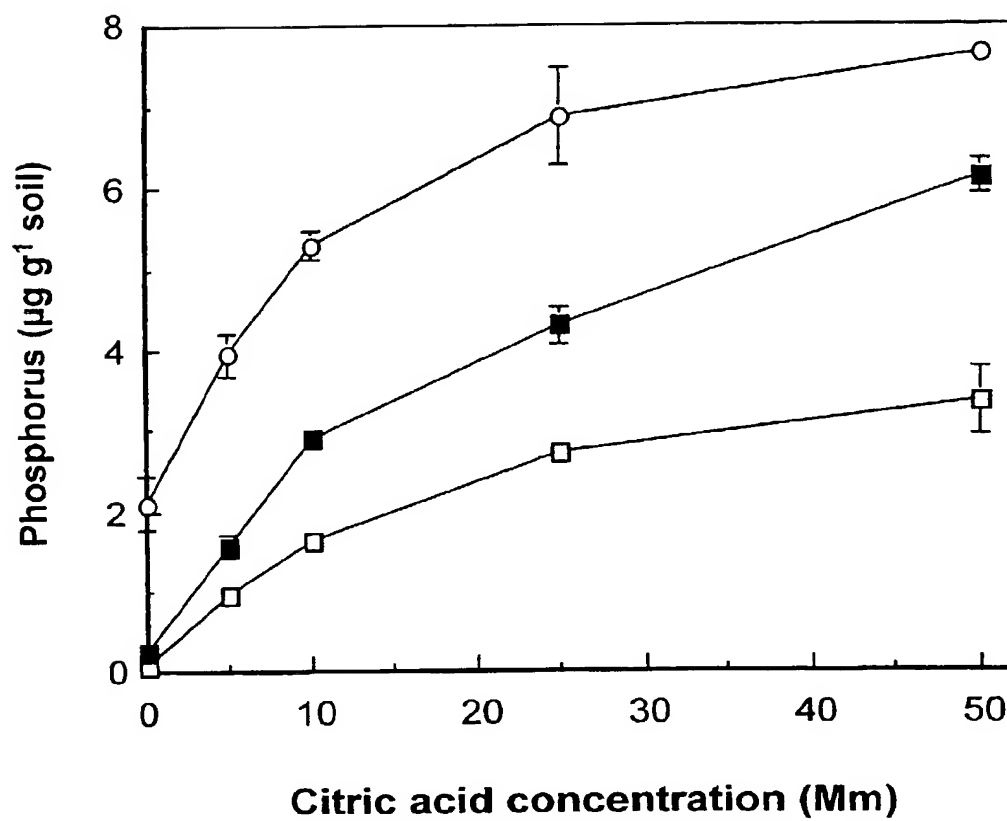


FIGURE 15

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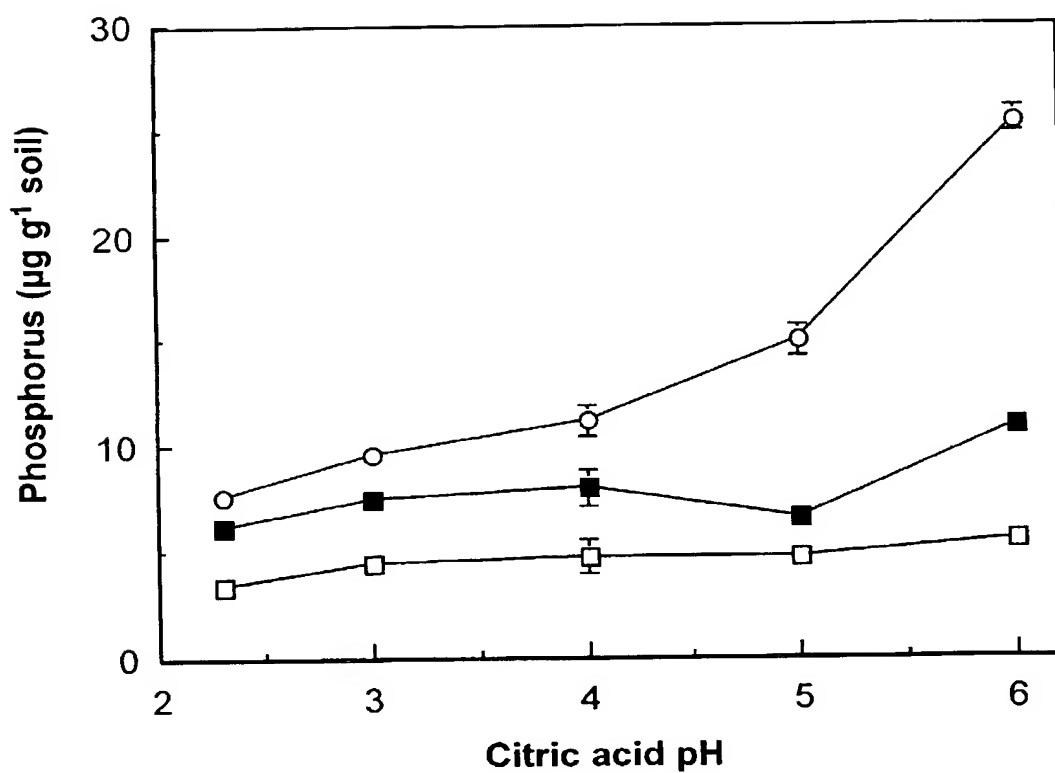


FIGURE 16

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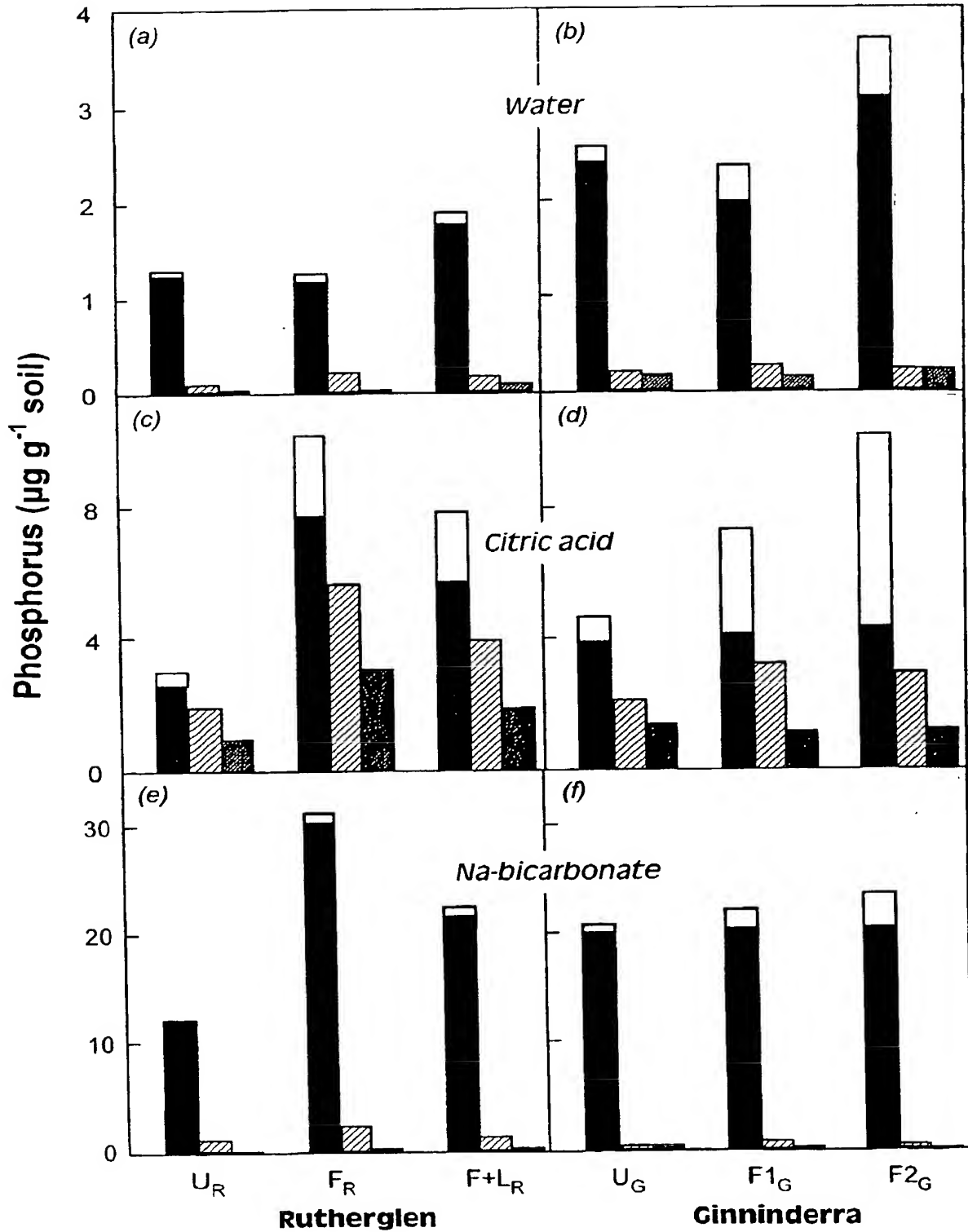


FIGURE 17

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<110> Richardson, Alan E.
Hayes, Julie E.
Simpson, Richard J.

<120> Expression of phytase in plants
as a method of modifying plant productivity

<130> 37-02

<140> US 10/089,364

<141> 2002-03-25

<150> PCT/AU00/01183

<151> 2000-09-23

<150> AU PQ3049

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<160> 18

<170> PatentIn Ver. 2.0

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Asp Gln Gly Tyr Gln Cys Phe Ser Glu Thr Ser His Leu Trp Gly Gln	
20 25 30	
tac gcg ccc ttc ttt tct ctg gca aac aaa tcg gcc atc tcc cct gat	144
Tyr Ala Pro Phe Phe Ser Leu Ala Asn Lys Ser Ala Ile Ser Pro Asp	
35 40 45	
gtt cct gcc gga tgc cat gtc act ttc gcc cag gtt ctc tcc cgc cat	192
Val Pro Ala Gly Cys His Val Thr Phe Ala Gln Val Leu Ser Arg His	
50 55 60	
gga gca cgg tat ccg acc gac tcc aag ggc aag aaa tac tcc gct ctc	240
Gly Ala Arg Tyr Pro Thr Asp Ser Lys Gly Lys Lys Tyr Ser Ala Leu	
65 70 75 80	
atc gag gag atc cag cag aac gcg aca acc ttc gag ggg aaa tat gcc	288
Ile Glu Glu Ile Gln Gln Asn Ala Thr Thr Phe Glu Gly Lys Tyr Ala	
85 90 95	

Tyr Glu Ser Leu Thr Arg Asn Ile Val Pro Phe Ile Arg Ser Ser Gly
 130 135 140
 Ser Asn Arg Val Ile Ala Ser Gly Asn Lys Phe Ile Glu Gly Phe Gln
 145 150 155 160
 Ser Thr Lys Leu Lys Asp Pro Arg Ala Gln Pro Gly Gln Ser Ser Pro
 165 170 175
 Lys Ile Asp Val Val Ile Ser Glu Ala Ser Thr Ser Asn Asn Thr Leu
 180 185 190
 Asp Pro Gly Thr Cys Thr Val Phe Glu Asp Ser Glu Leu Ala Asp Asp
 195 200 205
 Ile Glu Ala Asn Phe Thr Ala Thr Phe Val Pro Ser Ile Arg Gln Arg
 210 215 220
 Leu Glu Asn Asp Leu Ser Gly Val Ser Leu Thr Asp Thr Glu Val Thr
 225 230 235 240
 Tyr Leu Met Asp Met Cys Ser Phe Asp Thr Ile Ser Thr Ser Thr Val
 245 250 255
 Asp Thr Lys Leu Ser Pro Phe Cys Asp Leu Phe Thr His Glu Glu Trp
 260 265 270
 Ile Asn Tyr Asp Tyr Leu Gln Ser Pro Asn Lys Tyr Tyr Gly His Gly
 275 280 285
 Ala Gly Asn Pro Leu Gly Pro Thr Gln Gly Val Gly Tyr Ala Asn Glu
 290 295 300
 Leu Ile Ala Arg Leu Thr His Ser Pro Val His Asp Asp Thr Ser Ser
 305 310 315 320
 Asn His Thr Leu Asp Ser Asn Pro Ala Thr Phe Pro Leu Asn Ser Thr
 325 330 335
 Leu Tyr Ala Asp Phe Ser His Asp Asn Gly Ile Ile Ser Ile Leu Phe
 340 345 350
 Ala Leu Gly Leu Tyr Asn Gly Thr Lys Pro Leu Ser Ser Thr Thr Ala
 355 360 365
 Glu Asn Ile Thr Gln Thr Asp Gly Phe Ser Ser Ala Trp Thr Val Pro
 370 375 380
 Phe Ala Ser Arg Met Tyr Val Glu Met Met Gln Cys Gln Ser Glu Gln
 385 390 395 400
 Glu Pro Leu Val Arg Val Leu Val Asn Asp Arg Val Val Pro Leu His
 405 410 415
 Gly Cys Pro Val Asp Ala Leu Gly Arg Cys Thr Arg Asp Ser Phe Val
 420 425 430

Lys Gly Leu Ser Phe Ala Arg Ser Gly Gly Asp Trp Ala Glu Cys Phe
435 440 445

Ala

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<213> Aspergillus niger

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gat cag ggg tat caa tgc ttc tcc gag act tcg cat ctt tgg ggt caa 96
Asp Gln Gly Tyr Gln Cys Phe Ser Glu Thr Ser His Leu Trp Gly Gln
20 25 30
tac gca ccg ttc ttc tct ctg gca aac gaa tcg gtc atc tcc cct gag 144
Tyr Ala Pro Phe Phe Ser Leu Ala Asn Glu Ser Val Ile Ser Pro Glu
35 40 45
gtg ccc gcc gga tgc aga gtc act ttc gct cag gtc ctc tcc cgt cat 192
Val Pro Ala Gly Cys Arg Val Thr Phe Ala Gln Val Leu Ser Arg His
50 55 60
gga gcg cgg tat ccg acc gac tcc aag ggc aag aaa tac tcc gct ctc 240
Gly Ala Arg Tyr Pro Thr Asp Ser Lys Gly Lys Lys Tyr Ser Ala Leu
65 70 75 80
att gag gag atc cag cag aac gcg acc acc ttt gac gga aaa tat gcc 288
Ile Glu Glu Ile Gln Gln Asn Ala Thr Thr Phe Asp Gly Lys Tyr Ala
85 90 95
ttc ctg aag aca tac aac tac agc ttg ggt gca gat gac ctg act ccc 336
Phe Leu Lys Thr Tyr Asn Tyr Ser Leu Gly Ala Asp Asp Leu Thr Pro
100 105 110
ttc gga gaa cag gag cta gtc aac tcc ggc atc aag ttc tac cag cgg 384
Phe Gly Glu Gln Glu Leu Val Asn Ser Gly Ile Lys Phe Tyr Gln Arg
115 120 125
tac gaa tcg ctc aca agg aac atc gtt cca ttc atc cga tcc tct ggc 432
Tyr Glu Ser Leu Thr Arg Asn Ile Val Pro Phe Ile Arg Ser Ser Gly
130 135 140
tcc agc cgc gtg atc gcc tcc ggc aag aaa ttc atc gag ggc ttc cag 480
Ser Ser Arg Val Ile Ala Ser Gly Lys Lys Phe Ile Glu Gly Phe Gln
145 150 155 160
agc acc aag ctg aag gat cct cgt gcc cag ccc ggc caa tcg tcg ccc 528
Ser Thr Lys Leu Lys Asp Pro Arg Ala Gln Pro Gly Gln Ser Ser Pro
165 170 175

aag atc gac gtg gtc att tcc gag gcc agc tca tcc aac aac act ctc	576
Lys Ile Asp Val Val Ile Ser Glu Ala Ser Ser Ser Asn Asn Thr Leu	
180 185 190	
gac cca ggc acc tgc act gtc ttc gaa gac agc gaa ttg gcc gat acc	624
Asp Pro Gly Thr Cys Thr Val Phe Glu Asp Ser Glu Leu Ala Asp Thr	
195 200 205	
gtc gaa gcc aat ttc acc gcc acg ttc gtc ccc tcc att cgt caa cgt	672
Val Glu Ala Asn Phe Thr Ala Thr Phe Val Pro Ser Ile Arg Gln Arg	
210 215 220	
ctg gag aac gac ctg tcc ggt gtg act ctc aca gac aca gaa gtg acc	720
Leu Glu Asn Asp Leu Ser Gly Val Thr Leu Thr Asp Thr Glu Val Thr	
225 230 235 240	
tac ctc atg gac atg tgc tcc ttc gac acc atc tcc acc agc acc gtc	768
Tyr Leu Met Asp Met Cys Ser Phe Asp Thr Ile Ser Thr Ser Thr Val	
245 250 255	
gac acc aag ctg tcc ccc ttc tgt gac ctg ttc acc cat gac gaa tgg	816
Asp Thr Lys Leu Ser Pro Phe Cys Asp Leu Phe Thr His Asp Glu Trp	
260 265 270	
atc aac tac gac tac ctc cag tcc ttg aaa aag tat tac ggc cat ggt	864
Ile Asn Tyr Asp Tyr Leu Gln Ser Leu Lys Lys Tyr Tyr Gly His Gly	
275 280 285	
gca ggt aac ccg ctc ggc ccg acc cag ggc gtc ggc tac gct aac gag	912
Ala Gly Asn Pro Leu Gly Pro Thr Gln Gly Val Gly Tyr Ala Asn Glu	
290 295 300	
ctc atc gcc cgt ctg acc cac tcg cct gtc cac gat gac acc agt tcc	960
Leu Ile Ala Arg Leu Thr His Ser Pro Val His Asp Asp Thr Ser Ser	
305 310 315 320	
aac cac act ttg gac tcg agc ccg gct acc ttt ccg ctc aac tct act	1008
Asn His Thr Leu Asp Ser Ser Pro Ala Thr Phe Pro Leu Asn Ser Thr	
325 330 335	
ctc tac gcg gac ttt tcg cat gac aac ggc atc atc tcc att ctc ttt	1056
Leu Tyr Ala Asp Phe Ser His Asp Asn Gly Ile Ile Ser Ile Leu Phe	
340 345 350	
gct tta ggt ctg tac aac ggc act aag ccg cta tct acc acg acc gtg	1104
Ala Leu Gly Leu Tyr Asn Gly Thr Lys Pro Leu Ser Thr Thr Val	
355 360 365	
gag aat atc acc cag aca gat gga ttc tcg tct gct tgg acg gtt ccg	1152
Glu Asn Ile Thr Gln Thr Asp Gly Phe Ser Ser Ala Trp Thr Val Pro	
370 375 380	
ttt gct tcg cgt ttg tac gtc gag atg atg cag tgt cag gcg gag cag	1200
Phe Ala Ser Arg Leu Tyr Val Glu Met Met Gln Cys Gln Ala Glu Gln	
385 390 395 400	
gag ccg ctg gtc cgt gtc ttg gtt aat gat cgc gtt gtc ccg ctg cat	1248
Glu Pro Leu Val Arg Val Leu Val Asn Asp Arg Val Val Pro Leu His	
405 410 415	

ggg tgt ccg gtt gat gct ttg ggg aga tgt acc cgg gat agc ttt gtg 1296
Gly Cys Pro Val Asp Ala Leu Gly Arg Cys Thr Arg Asp Ser Phe Val
420 425 430

agg ggg ttg agc ttt gct aga tct ggg ggt gat tgg gcg gag tgt ttt 1344
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gct tag 1350
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Asp Gln Gly Tyr Gln Cys Phe Ser Glu Thr Ser His Leu Trp Gly Gln
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Tyr Ala Pro Phe Phe Ser Leu Ala Asn Glu Ser Val Ile Ser Pro Glu
35 40 45

Val Pro Ala Gly Cys Arg Val Thr Phe Ala Gln Val Leu Ser Arg His
50 55 60

Gly Ala Arg Tyr Pro Thr Asp Ser Lys Gly Lys Lys Tyr Ser Ala Leu
65 70 75 80

Ile Glu Glu Ile Gln Gln Asn Ala Thr Thr Phe Asp Gly Lys Tyr Ala
85 90 95

Phe Leu Lys Thr Tyr Asn Tyr Ser Leu Gly Ala Asp Asp Leu Thr Pro
100 105 110

Phe Gly Glu Gln Glu Leu Val Asn Ser Gly Ile Lys Phe Tyr Gln Arg
115 120 125

Tyr Glu Ser Leu Thr Arg Asn Ile Val Pro Phe Ile Arg Ser Ser Gly
130 135 140

Ser Ser Arg Val Ile Ala Ser Gly Lys Lys Phe Ile Glu Gly Phe Gln
145 150 155 160

Ser Thr Lys Leu Lys Asp Pro Arg Ala Gln Pro Gly Gln Ser Ser Pro
165 170 175

Lys Ile Asp Val Val Ile Ser Glu Ala Ser Ser Ser Asn Asn Thr Leu
180 185 190

Asp Pro Gly Thr Cys Thr Val Phe Glu Asp Ser Glu Leu Ala Asp Thr
195 200 205

Val Glu Ala Asn Phe Thr Ala Thr Phe Val Pro Ser Ile Arg Gln Arg
210 215 220

ttg ctt gta gta ttg gtg tca ctc aat ttg gct tcc gaa acc aca gct 96
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 <212> PRT
 <213> Daucus carota

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 <213> Lupinus luteus

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 gtg ttt tgc gat gga ggg aag acg agt agt ttt gtt agg gaa tct 93
 Val Phe Cys Asp Gly Gly Lys Thr Ser Ser Phe Val Arg Glu Ser
 20 25 30

<210> 8
 <211> 31
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 <213> Lupinus luteus

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 <212> DNA
 <213> Artificial Sequence

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 ext::phyA-2 gene

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ttg ctt gta gta ttg gtg tca ctc aat ttg gct tcc gaa acc aca gct 96
Leu Leu Val Val Leu Val Ser Leu Asn Leu Ala Ser Glu Thr Thr Ala
          20           25           30

gcc atg ctg gca gtc ccc gcc tcg aga aat caa tcc act tgc gat acg 144
Ala Met Leu Ala Val Pro Ala Ser Arg Asn Gln Ser Thr Cys Asp Thr
          35           40           45

gtc gat cag ggg tat caa tgc ttc tcg gag act tcg cat ctt tgg ggc 192
Val Asp Gln Gly Tyr Gln Cys Phe Ser Glu Thr Ser His Leu Trp Gly
          50           55           60

caa tac gcg ccc ttc ttt tct ctg gca aac aaa tcg gcc atc tcc cct 240
Gln Tyr Ala Pro Phe Phe Ser Leu Ala Asn Lys Ser Ala Ile Ser Pro
          65           70           75

gat gtt cct gcc gga tgc cat gtc act ttc gcc cag gtt ctc tcc cgc 288
Asp Val Pro Ala Gly Cys His Val Thr Phe Ala Gln Val Leu Ser Arg
          85           90           95

cat gga gca cgg tat ccg acc gac tcc aag ggc aag aaa tac tcc gct 336
His Gly Ala Arg Tyr Pro Thr Asp Ser Lys Gly Lys Lys Tyr Ser Ala
          100          105          110

ctc atc gag gag atc cag cag aac gcg aca acc ttc gag ggg aaa tat 384
Leu Ile Glu Glu Ile Gln Gln Asn Ala Thr Thr Phe Glu Gly Lys Tyr
          115          120          125

gcc ttc ctg aag aca tac aac tac agc ctg ggc gcg gat gat ctg act 432
Ala Phe Leu Lys Thr Tyr Asn Tyr Ser Leu Gly Ala Asp Asp Leu Thr
          130          135          140

ccc ttc gga gag cag gag ctg gtc aac tcc ggc gtc aag ttc tac cag 480
Pro Phe Gly Glu Gln Glu Leu Val Asn Ser Gly Val Lys Phe Tyr Gln
          145          150          155

cga tac gaa tcg ctc aca aga aac att gtc ccg ttc atc cga tcc tca 528
Arg Tyr Glu Ser Leu Thr Arg Asn Ile Val Pro Phe Ile Arg Ser Ser
          165          170          175

ggc tcc aac cgc gtg att gcc tct ggc aat aaa ttc atc gag ggc ttc 576
Gly Ser Asn Arg Val Ile Ala Ser Gly Asn Lys Phe Ile Glu Gly Phe
          180          185          190

cag agc act aag ctg aag gat cct cgt gcc cag ccc ggc caa tcg tcg 624
Gln Ser Thr Lys Leu Lys Asp Pro Arg Ala Gln Pro Gly Gln Ser Ser
          195          200          205

ccc aag atc gac gtg gtc att tca gag gcc agc aca tcc aac aac act 672
Pro Lys Ile Asp Val Val Ile Ser Glu Ala Ser Thr Ser Asn Asn Thr
          210          215          220

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gac atc gaa gcc aat ttc acc gcc acg ttc gtc ccc tcc att cgt caa	768
Asp Ile Glu Ala Asn Phe Thr Ala Thr Phe Val Pro Ser Ile Arg Gln	
245 250 255	
cgt ctg gag aat gac ttg tct ggc gtg tct ctc acg gac aca gaa gtg	816
Arg Leu Glu Asn Asp Leu Ser Gly Val Ser Leu Thr Asp Thr Glu Val	
260 265 270	
acc tac ctc atg gac atg tgc tcc ttc gac acc atc tcc acc agc acc	864
Thr Tyr Leu Met Asp Met Cys Ser Phe Asp Thr Ile Ser Thr Ser Thr	
275 280 285	
gtc gac acc aag ctg tcc ccc ttc tgt gac ctg ttc acc cat gaa gaa	912
Val Asp Thr Lys Leu Ser Pro Phe Cys Asp Leu Phe Thr His Glu Glu	
290 295 300	
tgg atc aac tac gac tac ctc cag tcc ccg aac aaa tac tac ggc cat	960
Trp Ile Asn Tyr Asp Tyr Leu Gln Ser Pro Asn Lys Tyr Tyr Gly His	
305 310 315 320	
ggc gca ggt aac ccg ctc ggc ccg acc cag ggc gtc ggc tac gct aac	1008
Gly Ala Gly Asn Pro Leu Gly Pro Thr Gln Gly Val Gly Tyr Ala Asn	
325 330 335	
gag ctc atc gcc cgt ctc acc cac tcg cct gtc cac gat gac acc agc	1056
Glu Leu Ile Ala Arg Leu Thr His Ser Pro Val His Asp Asp Thr Ser	
340 345 350	
tcc aac cac aca ttg gac tcc aac ccg gct act ttc ccg ctc aac tcc	1104
Ser Asn His Thr Leu Asp Ser Asn Pro Ala Thr Phe Pro Leu Asn Ser	
355 360 365	
act ctc tat gcg gac ttt tcg cat gat aac ggc atc atc tct atc ctc	1152
Thr Leu Tyr Ala Asp Phe Ser His Asp Asn Gly Ile Ile Ser Ile Leu	
370 375 380	
ttt gct ttg ggt ctg tac aac ggc acc aag ccg ctg tct tcc acg acc	1200
Phe Ala Leu Gly Leu Tyr Asn Gly Thr Lys Pro Leu Ser Ser Thr Thr	
385 390 395 400	
gcg gag aat atc acc cag acc gat ggg ttc tca tct gcc tgg acg gtt	1248
Ala Glu Asn Ile Thr Gln Thr Asp Gly Phe Ser Ser Ala Trp Thr Val	
405 410 415	
cct ttc gcg tcg cgc atg tac gtc gag atg atg caa tgc cag tcc gag	1296
Pro Phe Ala Ser Arg Met Tyr Val Glu Met Met Gln Cys Gln Ser Glu	
420 425 430	
cag gag cct ttg gtc cgt gtc ttg gtt aat gat cgt gtt gtt ccg ctg	1344
Gln Glu Pro Leu Val Arg Val Leu Val Asn Asp Arg Val Val Pro Leu	
435 440 445	
cat ggc tgt ccg gtt gat gct ttg gga aga tgt acg cgg gat agc ttc	1392
His Gly Cys Pro Val Asp Ala Leu Gly Arg Cys Thr Arg Asp Ser Phe	
450 455 460	

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gtg aag ggg ttg agc ttt gcc aga tct ggc ggt gat tgg gcg gag tgt      1440
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Phe Ala

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      20              25              30

Ala Met Leu Ala Val Pro Ala Ser Arg Asn Gln Ser Thr Cys Asp Thr
      35              40              45

Val Asp Gln Gly Tyr Gln Cys Phe Ser Glu Thr Ser His Leu Trp Gly
      50              55              60

Gln Tyr Ala Pro Phe Phe Ser Leu Ala Asn Lys Ser Ala Ile Ser Pro
      65              70              75              80

Asp Val Pro Ala Gly Cys His Val Thr Phe Ala Gln Val Leu Ser Arg
      85              90              95

His Gly Ala Arg Tyr Pro Thr Asp Ser Lys Gly Lys Lys Tyr Ser Ala
      100             105             110

Leu Ile Glu Glu Ile Gln Gln Asn Ala Thr Thr Phe Glu Gly Lys Tyr
      115             120             125

Ala Phe Leu Lys Thr Tyr Asn Tyr Ser Leu Gly Ala Asp Asp Leu Thr
      130             135             140

Pro Phe Gly Glu Gln Glu Leu Val Asn Ser Gly Val Lys Phe Tyr Gln
      145             150             155             160

Arg Tyr Glu Ser Leu Thr Arg Asn Ile Val Pro Phe Ile Arg Ser Ser
      165             170             175

Gly Ser Asn Arg Val Ile Ala Ser Gly Asn Lys Phe Ile Glu Gly Phe
      180             185             190

Gln Ser Thr Lys Leu Lys Asp Pro Arg Ala Gln Pro Gly Gln Ser Ser
      195             200             205

Pro Lys Ile Asp Val Val Ile Ser Glu Ala Ser Thr Ser Asn Asn Thr
      210             215             220

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<220>

<221> CDS

<222> (1)..(1446)

<400> 11

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  1           5           10           15

ttg ctt gta gta ttg gtg tca ctc aat ttg gct tcc gaa acc aca gct 96
Leu Leu Val Val Leu Val Ser Leu Asn Leu Ala Ser Glu Thr Thr Ala
          20           25           30

gcc atg ctg gca gtc ccc gcc tcg aga aat caa tcc agt tgc gat acg 144
Ala Met Leu Ala Val Pro Ala Ser Arg Asn Gln Ser Ser Cys Asp Thr
          35           40           45

gtc gat cag ggg tat caa tgc ttc tcc gag act tcg cat ctt tgg ggt 192
Val Asp Gln Gly Tyr Gln Cys Phe Ser Glu Thr Ser His Leu Trp Gly
          50           55           60

caa tac gca ccg ttc ttc tct ctg gca aac gaa tcg gtc atc tcc cct 240
Gln Tyr Ala Pro Phe Phe Ser Leu Ala Asn Glu Ser Val Ile Ser Pro
          65           70           75

gag gtg ccc gcc gga tgc aga gtc act ttc gct cag gtc ctc tcc cgt 288
Glu Val Pro Ala Gly Cys Arg Val Thr Phe Ala Gln Val Leu Ser Arg
          85           90           95

cat gga gcg cgg tat ccg acc gac tcc aag ggc aag aaa tac tcc gct 336
His Gly Ala Arg Tyr Pro Thr Asp Ser Lys Gly Lys Lys Tyr Ser Ala
          100          105          110

ctc att gag gag atc cag cag aac gcg acc acc ttt gac gga aaa tat 384
Leu Ile Glu Glu Ile Gln Gln Asn Ala Thr Thr Phe Asp Gly Lys Tyr
          115          120          125

gcc ttc ctg aag aca tac aac tac agc ttg ggt gca gat gac ctg act 432
Ala Phe Leu Lys Thr Tyr Asn Tyr Ser Leu Gly Ala Asp Asp Leu Thr
          130          135          140

ccc ttc gga gaa cag gag cta gtc aac tcc ggc atc aag ttc tac cag 480
Pro Phe Gly Glu Gln Glu Leu Val Asn Ser Gly Ile Lys Phe Tyr Gln
          145          150          155

cgg tac gaa tcg ctc aca agg aac atc gtt cca ttc atc cga tcc tct 528
Arg Tyr Glu Ser Leu Thr Arg Asn Ile Val Pro Phe Ile Arg Ser Ser
          165          170          175

ggc tcc agc cgc gtg atc gcc tcc ggc aag aaa ttc atc gag ggc ttc 576
Gly Ser Ser Arg Val Ile Ala Ser Gly Lys Lys Phe Ile Glu Gly Phe
          180          185          190

cag agc acc aag ctg aag gat cct cgt gcc cag ccc ggc caa tcg tcg 624
Gln Ser Thr Lys Leu Lys Asp Pro Arg Ala Gln Pro Gly Gln Ser Ser
          195          200          205

ccc aag atc gac gtg gtc att tcc gag gcc agc tca tcc aac aac act 672
Pro Lys Ile Asp Val Val Ile Ser Glu Ala Ser Ser Ser Asn Asn Thr
          210          215          220

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ctc gac cca ggc acc tgc act gtc ttc gaa gac agc gaa ttg gcc gat	720
Leu Asp Pro Gly Thr Cys Thr Val Phe Glu Asp Ser Glu Leu Ala Asp	
225 230 235 240	
acc gtc gaa gcc aat ttc acc gcc acg ttc gtc ccc tcc att cgt caa	768
Thr Val Glu Ala Asn Phe Thr Ala Thr Phe Val Pro Ser Ile Arg Gln	
245 250 255	
cgt ctg gag aac gac ctg tcc ggt gtg act ctc aca gac aca gaa gtg	816
Arg Leu Glu Asn Asp Leu Ser Gly Val Thr Leu Thr Asp Thr Glu Val	
260 265 270	
acc tac ctc atg gac atg tgc tcc ttc gac acc atc tcc acc agc acc	864
Thr Tyr Leu Met Asp Met Cys Ser Phe Asp Thr Ile Ser Thr Ser Thr	
275 280 285	
gtc gac acc aag ctg tcc ccc ttc tgt gac ctg ttc acc cat gac gaa	912
Val Asp Thr Lys Leu Ser Pro Phe Cys Asp Leu Phe Thr His Asp Glu	
290 295 300	
tgg atc aac tac gac tac ctc cag tcc ttg aaa aag tat tac ggc cat	960
Trp Ile Asn Tyr Asp Tyr Leu Gln Ser Leu Lys Lys Tyr Tyr Gly His	
305 310 315 320	
ggt gca ggt aac ccg ctc ggc ccg acc cag ggc gtc ggc tac gct aac	1008
Gly Ala Gly Asn Pro Leu Gly Pro Thr Gln Gly Val Gly Tyr Ala Asn	
325 330 335	
gag ctc atc gcc cgt ctg acc cac tcg cct gtc cac gat gac acc agt	1056
Glu Leu Ile Ala Arg Leu Thr His Ser Pro Val His Asp Asp Thr Ser	
340 345 350	
tcc aac cac act ttg gac tcg agc ccg gct acc ttt ccg ctc aac tct	1104
Ser Asn His Thr Leu Asp Ser Ser Pro Ala Thr Phe Pro Leu Asn Ser	
355 360 365	
act ctc tac gcg gac ttt tcg cat gac aac ggc atc atc tcc att ctc	1152
Thr Leu Tyr Ala Asp Phe Ser His Asp Asn Gly Ile Ile Ser Ile Leu	
370 375 380	
ttt gct tta ggt ctg tac aac ggc act aag ccg cta tct acc acg acc	1200
Phe Ala Leu Gly Leu Tyr Asn Gly Thr Lys Pro Leu Ser Thr Thr Thr	
385 390 395 400	
gtg gag aat atc acc cag aca gat gga ttc tcg tct gct tgg acg gtt	1248
Val Glu Asn Ile Thr Gln Thr Asp Gly Phe Ser Ser Ala Trp Thr Val	
405 410 415	
ccg ttt gct tcg cgt ttg tac gtc gag atg atg cag tgt cag gcg gag	1296
Pro Phe Ala Ser Arg Leu Tyr Val Glu Met Met Gln Cys Gln Ala Glu	
420 425 430	
cag gag ccg ctg gtc cgt gtc ttg gtt aat gat cgc gtt gtc ccg ctg	1344
Gln Glu Pro Leu Val Arg Val Leu Val Asn Asp Arg Val Val Pro Leu	
435 440 445	
cat ggg tgt ccg gtt gat gct ttg ggg aga tgt acc cgg gat agc ttt	1392
His Gly Cys Pro Val Asp Ala Leu Gly Arg Cys Thr Arg Asp Ser Phe	
450 455 460	

gtg agg ggg ttg agc ttt gct aga tct ggg ggt gat tgg gcg gag tgt 1440
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 Phe Ala

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 20 25 30
 Ala Met Leu Ala Val Pro Ala Ser Arg Asn Gln Ser Ser Cys Asp Thr
 35 40 45
 Val Asp Gln Gly Tyr Gln Cys Phe Ser Glu Thr Ser His Leu Trp Gly
 50 55 60
 Gln Tyr Ala Pro Phe Phe Ser Leu Ala Asn Glu Ser Val Ile Ser Pro
 65 70 75 80
 Glu Val Pro Ala Gly Cys Arg Val Thr Phe Ala Gln Val Leu Ser Arg
 85 90 95
 His Gly Ala Arg Tyr Pro Thr Asp Ser Lys Gly Lys Lys Tyr Ser Ala
 100 105 110
 Leu Ile Glu Glu Ile Gln Gln Asn Ala Thr Thr Phe Asp Gly Lys Tyr
 115 120 125
 Ala Phe Leu Lys Thr Tyr Asn Tyr Ser Leu Gly Ala Asp Asp Leu Thr
 130 135 140
 Pro Phe Gly Glu Gln Glu Leu Val Asn Ser Gly Ile Lys Phe Tyr Gln
 145 150 155 160
 Arg Tyr Glu Ser Leu Thr Arg Asn Ile Val Pro Phe Ile Arg Ser Ser
 165 170 175
 Gly Ser Ser Arg Val Ile Ala Ser Gly Lys Lys Phe Ile Glu Gly Phe
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 195 200 205
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 210 215 220

<400> 13
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<210> 14
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 <212> DNA
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<400> 14
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<210> 15
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 <213> Artificial Sequence

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 <212> DNA
 <213> Artificial Sequence

<220>
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 <223> Description of Artificial Sequence:ext::phytase
 junction

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<210> 18
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 <213> Artificial Sequence

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 <223> Description of Artificial Sequence:ext::phytase
 junction

$\frac{d^2}{dt^2} \left(\frac{1}{2} m \dot{x}^2 \right) = \frac{d}{dt} \left(m \dot{x} \frac{dx}{dt} \right) = m \frac{d^2 x}{dt^2} \frac{dx}{dt} + m \dot{x} \frac{d^2 x}{dt^2} = m \frac{d^2 x}{dt^2} \left(\frac{dx}{dt} + \dot{x} \right) = m \frac{d^2 x}{dt^2} \frac{dx}{dt}$

<400> 18
 Thr Ala Ala Met Leu Ala
 1 5
 18

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SEQUENCE LISTING

<110> Commonwealth Scientific and Industrial Research
 Organisation AND
 5 Australian Wool Research and Promotion Organisation

<120> Method of modifying plant productivity

<130> p:\oper\mro\phytase.pct
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<140> PCT/AU00/XXXXX
 <141> 2000-09-23

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 1 5 10 15

gat cag ggg tat caa tgc ttc tcg gag act tcg cat ctt tgg ggc caa 96
 Asp Gln Gly Tyr Gln Cys Phe Ser Glu Thr Ser His Leu Trp Gly Gln
 20 25 30

tac gcg ccc ttc ttt tct ctg gca aac aaa tcg gcc atc tcc cct gat 144
 40 Tyr Ala Pro Phe Phe Ser Leu Ala Asn Lys Ser Ala Ile Ser Pro Asp
 35 40 45

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gtt cct gcc gga tgc cat gtc act ttc gcc cag gtt ctc tcc cgc cat 192
Val Pro Ala Gly Cys His Val Thr Phe Ala Gln Val Leu Ser Arg His
      50              55              60

5  gga gca cgg tat ccg acc gac tcc aag ggc aag aaa tac tcc gct ctc 240
   Gly Ala Arg Tyr Pro Thr Asp Ser Lys Gly Lys Lys Tyr Ser Ala Leu
      65              70              75              80

   atc gag gag atc cag cag aac gcg aca acc ttc gag ggg aaa tat gcc 288
10  Ile Glu Glu Ile Gln Gln Asn Ala Thr Thr Phe Glu Gly Lys Tyr Ala
      85              90              95

   ttc ctg aag aca tac aac tac agc ctg ggc gcg gat gat ctg act ccc 336
15  Phe Leu Lys Thr Tyr Asn Tyr Ser Leu Gly Ala Asp Asp Leu Thr Pro
      100             105             110

   ttc gga gag cag gag ctg gtc aac tcc ggc gtc aag ttc tac cag cga 384
   Phe Gly Glu Gln Glu Leu Val Asn Ser Gly Val Lys Phe Tyr Gln Arg
      115             120             125

20  tac gaa tcg ctc aca aga aac att gtc ccg ttc atc cga tcc tca ggc 432
   Tyr Glu Ser Leu Thr Arg Asn Ile Val Pro Phe Ile Arg Ser Ser Gly
      130             135             140

25  tcc aac cgc gtg att gcc tct ggc aat aaa ttc atc gag ggc ttc cag 480
   Ser Asn Arg Val Ile Ala Ser Gly Asn Lys Phe Ile Glu Gly Phe Gln
      145             150             155             160

   agc act aag ctg aag gat cct cgt gcc cag ccc ggc caa tcg tcg ccc 528
30  Ser Thr Lys Leu Lys Asp Pro Arg Ala Gln Pro Gly Gln Ser Ser Pro
      165             170             175

   aag atc gac gtg gtc att tca gag gcc agc aca tcc aac aac act ctc 576
35  Lys Ile Asp Val Val Ile Ser Glu Ala Ser Thr Ser Asn Asn Thr Leu
      180             185             190

   gat ccg ggc acc tgc acc gtt ttc gaa gat agc gaa ttg gcc gat gac 624
   Asp Pro Gly Thr Cys Thr Val Phe Glu Asp Ser Glu Leu Ala Asp Asp
      195             200             205

40  atc gaa gcc aat ttc acc gcc acg ttc gtc ccc tcc att cgt caa cgt 672
   Ile Glu Ala Asn Phe Thr Ala Thr Phe Val Pro Ser Ile Arg Gln Arg

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	210	215	220	
	ctg gag aat gac ttg tct ggc gtg tct ctc acg gac aca gaa gtg acc			720
	Leu Glu Asn Asp Leu Ser Gly Val Ser Leu Thr Asp Thr Glu Val Thr			
5	225	230	235	240
	tac ctc atg gac atg tgc tcc ttc gac acc atc tcc acc agc acc gtc			768
	Tyr Leu Met Asp Met Cys Ser Phe Asp Thr Ile Ser Thr Ser Thr Val			
		245	250	255
10				
	gac acc aag ctg tcc ccc ttc tgt gac ctg ttc acc cat gaa gaa tgg			816
	Asp Thr Lys Leu Ser Pro Phe Cys Asp Leu Phe Thr His Glu Glu Trp			
		260	265	270
15				
	atc aac tac gac tac ctc cag tcc ccg aac aaa tac tac ggc cat ggc			864
	Ile Asn Tyr Asp Tyr Leu Gln Ser Pro Asn Lys Tyr Tyr Gly His Gly			
		275	280	285
	gca ggt aac ccg ctc ggc ccg acc cag ggc gtc ggc tac gct aac gag			912
20	Ala Gly Asn Pro Leu Gly Pro Thr Gln Gly Val Gly Tyr Ala Asn Glu			
		290	295	300
	ctc atc gcc cgt ctc acc cac tcg cct gtc cac gat gac acc agc tcc			960
	Leu Ile Ala Arg Leu Thr His Ser Pro Val His Asp Asp Thr Ser Ser			
25	305	310	315	320
	aac cac aca ttg gac tcc aac ccg gct act ttc ccg ctc aac tcc act			1008
	Asn His Thr Leu Asp Ser Asn Pro Ala Thr Phe Pro Leu Asn Ser Thr			
		325	330	335
30				
	ctc tat gcg gac ttt tcg cat gat aac ggc atc atc tct atc ctc ttt			1056
	Leu Tyr Ala Asp Phe Ser His Asp Asn Gly Ile Ile Ser Ile Leu Phe			
		340	345	350
35				
	gct ttg ggt ctg tac aac ggc acc aag ccg ctg tct tcc acg acc gcg			1104
	Ala Leu Gly Leu Tyr Asn Gly Thr Lys Pro Leu Ser Ser Thr Thr Ala			
		355	360	365
	gag aat atc acc cag acc gat ggg ttc tca tct gcc tgg acg gtt cct			1152
40	Glu Asn Ile Thr Gln Thr Asp Gly Phe Ser Ser Ala Trp Thr Val Pro			
		370	375	380

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    ttc gcg tcg cgc atg tac gtc gag atg atg caa tgc cag tcc gag cag    1200
    Phe Ala Ser Arg Met Tyr Val Glu Met Met Gln Cys Gln Ser Glu Gln
    385                      390                      395                      400

5   gag cct ttg gtc cgt gtc ttg gtt aat gat cgt gtt gtt ccg ctg cat    1248
    Glu Pro Leu Val Arg Val Leu Val Asn Asp Arg Val Val Pro Leu His
                                405                      410                      415

    ggc tgt ccg gtt gat gct ttg gga aga tgt acg cgg gat agc ttc gtg    1296
10  Gly Cys Pro Val Asp Ala Leu Gly Arg Cys Thr Arg Asp Ser Phe Val
                                420                      425                      430

    aag ggg ttg agc ttt gcc aga tct ggc ggt gat tgg gcg gag tgt ttt    1344
15  Lys Gly Leu Ser Phe Ala Arg Ser Gly Gly Asp Trp Ala Glu Cys Phe
                                435                      440                      445

    gct tag                                                                1350
    Ala

20
    <210> 2
    <211> 449
    <212> PRT
    <213> Aspergillus niger

25
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        1                      5                      10                      15

30  Asp Gln Gly Tyr Gln Cys Phe Ser Glu Thr Ser His Leu Trp Gly Gln
        20                      25                      30

    Tyr Ala Pro Phe Phe Ser Leu Ala Asn Lys Ser Ala Ile Ser Pro Asp
        35                      40                      45

35  Val Pro Ala Gly Cys His Val Thr Phe Ala Gln Val Leu Ser Arg His
        50                      55                      60

    Gly Ala Arg Tyr Pro Thr Asp Ser Lys Gly Lys Lys Tyr Ser Ala Leu
40  65                      70                      75                      80

    Ile Glu Glu Ile Gln Gln Asn Ala Thr Thr Phe Glu Gly Lys Tyr Ala

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	85	90	95
	Phe Leu Lys Thr Tyr Asn Tyr Ser Leu Gly Ala Asp Asp Leu Thr Pro		
	100	105	110
5	Phe Gly Glu Gln Glu Leu Val Asn Ser Gly Val Lys Phe Tyr Gln Arg		
	115	120	125
	Tyr Glu Ser Leu Thr Arg Asn Ile Val Pro Phe Ile Arg Ser Ser Gly		
10	130	135	140
	Ser Asn Arg Val Ile Ala Ser Gly Asn Lys Phe Ile Glu Gly Phe Gln		
	145	150	155
	Ser Thr Lys Leu Lys Asp Pro Arg Ala Gln Pro Gly Gln Ser Ser Pro		
15	165	170	175
	Lys Ile Asp Val Val Ile Ser Glu Ala Ser Thr Ser Asn Asn Thr Leu		
	180	185	190
20	Asp Pro Gly Thr Cys Thr Val Phe Glu Asp Ser Glu Leu Ala Asp Asp		
	195	200	205
	Ile Glu Ala Asn Phe Thr Ala Thr Phe Val Pro Ser Ile Arg Gln Arg		
25	210	215	220
	Leu Glu Asn Asp Leu Ser Gly Val Ser Leu Thr Asp Thr Glu Val Thr		
	225	230	235
	Tyr Leu Met Asp Met Cys Ser Phe Asp Thr Ile Ser Thr Ser Thr Val		
30	245	250	255
	Asp Thr Lys Leu Ser Pro Phe Cys Asp Leu Phe Thr His Glu Glu Trp		
	260	265	270
35	Ile Asn Tyr Asp Tyr Leu Gln Ser Pro Asn Lys Tyr Tyr Gly His Gly		
	275	280	285
	Ala Gly Asn Pro Leu Gly Pro Thr Gln Gly Val Gly Tyr Ala Asn Glu		
40	290	295	300
	Leu Ile Ala Arg Leu Thr His Ser Pro Val His Asp Asp Thr Ser Ser		

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305              310              315              320
Asn His Thr Leu Asp Ser Asn Pro Ala Thr Phe Pro Leu Asn Ser Thr
      325              330              335
5
Leu Tyr Ala Asp Phe Ser His Asp Asn Gly Ile Ile Ser Ile Leu Phe
      340              345              350
Ala Leu Gly Leu Tyr Asn Gly Thr Lys Pro Leu Ser Ser Thr Thr Ala
10      355              360              365
Glu Asn Ile Thr Gln Thr Asp Gly Phe Ser Ser Ala Trp Thr Val Pro
      370              375              380
15
Phe Ala Ser Arg Met Tyr Val Glu Met Met Gln Cys Gln Ser Glu Gln
385              390              395              400
Glu Pro Leu Val Arg Val Leu Val Asn Asp Arg Val Val Pro Leu His
      405              410              415
20
Gly Cys Pro Val Asp Ala Leu Gly Arg Cys Thr Arg Asp Ser Phe Val
      420              425              430
Lys Gly Leu Ser Phe Ala Arg Ser Gly Gly Asp Trp Ala Glu Cys Phe
25      435              440              445
Ala
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<213> Aspergillus niger
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Met Leu Ala Val Pro Ala Ser Arg Asn Gln Ser Ser Cys Asp Thr Val
48

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	1	5	10	15	
	gat cag ggg tat caa tgc ttc tcc gag act tcg cat ctt tgg ggt caa				96
	Asp Gln Gly Tyr Gln Cys Phe Ser Glu Thr Ser His Leu Trp Gly Gln				
5	20	25	30		
	tac gca ccg ttc ttc tct ctg gca aac gaa tcg gtc atc tcc cct gag				144
	Tyr Ala Pro Phe Phe Ser Leu Ala Asn Glu Ser Val Ile Ser Pro Glu				
	35	40	45		
10	gtg ccc gcc gga tgc aga gtc act ttc gct cag gtc ctc tcc cgt cat				192
	Val Pro Ala Gly Cys Arg Val Thr Phe Ala Gln Val Leu Ser Arg His				
	50	55	60		
15	gga gcg cgg tat ccg acc gac tcc aag ggc aag aaa tac tcc gct ctc				240
	Gly Ala Arg Tyr Pro Thr Asp Ser Lys Gly Lys Lys Tyr Ser Ala Leu				
	65	70	75	80	
	att gag gag atc cag cag aac gcg acc acc ttt gac gga aaa tat gcc				288
20	Ile Glu Glu Ile Gln Gln Asn Ala Thr Thr Phe Asp Gly Lys Tyr Ala				
	85	90	95		
	ttc ctg aag aca tac aac tac agc ttg ggt gca gat gac ctg act ccc				336
	Phe Leu Lys Thr Tyr Asn Tyr Ser Leu Gly Ala Asp Asp Leu Thr Pro				
25	100	105	110		
	ttc gga gaa cag gag cta gtc aac tcc ggc atc aag ttc tac cag cgg				384
	Phe Gly Glu Gln Glu Leu Val Asn Ser Gly Ile Lys Phe Tyr Gln Arg				
	115	120	125		
30	tac gaa tcg ctc aca agg aac atc gtt cca ttc atc cga tcc tct ggc				432
	Tyr Glu Ser Leu Thr Arg Asn Ile Val Pro Phe Ile Arg Ser Ser Gly				
	130	135	140		
35	tcc agc cgc gtg atc gcc tcc ggc aag aaa ttc atc gag ggc ttc cag				480
	Ser Ser Arg Val Ile Ala Ser Gly Lys Lys Phe Ile Glu Gly Phe Gln				
	145	150	155	160	
	agc acc aag ctg aag gat cct cgt gcc cag ccc ggc caa tcg tcg ccc				528
40	Ser Thr Lys Leu Lys Asp Pro Arg Ala Gln Pro Gly Gln Ser Ser Pro				
	165	170	175		

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aag atc gac gtg gtc att tcc gag gcc agc tca tcc aac aac act ctc 576
 Lys Ile Asp Val Val Ile Ser Glu Ala Ser Ser Ser Asn Asn Thr Leu
 180 185 190

5 gac cca ggc acc tgc act gtc ttc gaa gac agc gaa ttg gcc gat acc 624
 Asp Pro Gly Thr Cys Thr Val Phe Glu Asp Ser Glu Leu Ala Asp Thr
 195 200 205

10 gtc gaa gcc aat ttc acc gcc acg ttc gtc ccc tcc att cgt caa cgt 672
 Val Glu Ala Asn Phe Thr Ala Thr Phe Val Pro Ser Ile Arg Gln Arg
 210 215 220

15 ctg gag aac gac ctg tcc ggt gtg act ctc aca gac aca gaa gtg acc 720
 Leu Glu Asn Asp Leu Ser Gly Val Thr Leu Thr Asp Thr Glu Val Thr
 225 230 235 240

20 tac ctc atg gac atg tgc tcc ttc gac acc atc tcc acc agc acc gtc 768
 Tyr Leu Met Asp Met Cys Ser Phe Asp Thr Ile Ser Thr Ser Thr Val
 245 250 255

gac acc aag ctg tcc ccc ttc tgt gac ctg ttc acc cat gac gaa tgg 816
 Asp Thr Lys Leu Ser Pro Phe Cys Asp Leu Phe Thr His Asp Glu Trp
 260 265 270

25 atc aac tac gac tac ctc cag tcc ttg aaa aag tat tac ggc cat ggt 864
 Ile Asn Tyr Asp Tyr Leu Gln Ser Leu Lys Lys Tyr Tyr Gly His Gly
 275 280 285

30 gca ggt aac ccg ctc ggc ccg acc cag ggc gtc ggc tac gct aac gag 912
 Ala Gly Asn Pro Leu Gly Pro Thr Gln Gly Val Gly Tyr Ala Asn Glu
 290 295 300

35 ctc atc gcc cgt ctg acc cac tcg cct gtc cac gat gac acc agt tcc 960
 Leu Ile Ala Arg Leu Thr His Ser Pro Val His Asp Asp Thr Ser Ser
 305 310 315 320

40 aac cac act ttg gac tcg agc ccg gct acc ttt ccg ctc aac tct act 1008
 Asn His Thr Leu Asp Ser Ser Pro Ala Thr Phe Pro Leu Asn Ser Thr
 325 330 335

ctc tac gcg gac ttt tcg cat gac aac ggc atc atc tcc att ctc ttt 1056
 Leu Tyr Ala Asp Phe Ser His Asp Asn Gly Ile Ile Ser Ile Leu Phe

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	340	345	350	
	gct tta ggt ctg tac aac ggc act aag ccg cta tct acc acg acc gtg			1104
	Ala Leu Gly Leu Tyr Asn Gly Thr Lys Pro Leu Ser Thr Thr Thr Val			
5	355	360	365	
	gag aat atc acc cag aca gat gga ttc tcg tct gct tgg acg gtt ccg			1152
	Glu Asn Ile Thr Gln Thr Asp Gly Phe Ser Ser Ala Trp Thr Val Pro			
	370	375	380	
10	ttt gct tcg cgt ttg tac gtc gag atg atg cag tgt cag gcg gag cag			1200
	Phe Ala Ser Arg Leu Tyr Val Glu Met Met Gln Cys Gln Ala Glu Gln			
	385	390	395	400
15	gag ccg ctg gtc cgt gtc ttg gtt aat gat cgc gtt gtc ccg ctg cat			1248
	Glu Pro Leu Val Arg Val Leu Val Asn Asp Arg Val Val Pro Leu His			
	405	410	415	
	ggg tgt ccg gtt gat gct ttg ggg aga tgt acc ccg gat agc ttt gtg			1296
20	Gly Cys Pro Val Asp Ala Leu Gly Arg Cys Thr Arg Asp Ser Phe Val			
	420	425	430	
	agg ggg ttg agc ttt gct aga tct ggg ggt gat tgg gcg gag tgt ttt			1344
	Arg Gly Leu Ser Phe Ala Arg Ser Gly Gly Asp Trp Ala Glu Cys Phe			
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	20	25	30	

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Tyr Ala Pro Phe Phe Ser Leu Ala Asn Glu Ser Val Ile Ser Pro Glu
 35 40 45

Val Pro Ala Gly Cys Arg Val Thr Phe Ala Gln Val Leu Ser Arg His
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Gly Ala Arg Tyr Pro Thr Asp Ser Lys Gly Lys Lys Tyr Ser Ala Leu
 65 70 75 80

10 Ile Glu Glu Ile Gln Gln Asn Ala Thr Thr Phe Asp Gly Lys Tyr Ala
 85 90 95

Phe Leu Lys Thr Tyr Asn Tyr Ser Leu Gly Ala Asp Asp Leu Thr Pro
 100 105 110

15 Phe Gly Glu Gln Glu Leu Val Asn Ser Gly Ile Lys Phe Tyr Gln Arg
 115 120 125

Tyr Glu Ser Leu Thr Arg Asn Ile Val Pro Phe Ile Arg Ser Ser Gly
 20 130 135 140

Ser Ser Arg Val Ile Ala Ser Gly Lys Lys Phe Ile Glu Gly Phe Gln
 145 150 155 160

25 Ser Thr Lys Leu Lys Asp Pro Arg Ala Gln Pro Gly Gln Ser Ser Pro
 165 170 175

Lys Ile Asp Val Val Ile Ser Glu Ala Ser Ser Ser Asn Asn Thr Leu
 180 185 190

30 Asp Pro Gly Thr Cys Thr Val Phe Glu Asp Ser Glu Leu Ala Asp Thr
 195 200 205

Val Glu Ala Asn Phe Thr Ala Thr Phe Val Pro Ser Ile Arg Gln Arg
 35 210 215 220

Leu Glu Asn Asp Leu Ser Gly Val Thr Leu Thr Asp Thr Glu Val Thr
 225 230 235 240

40 Tyr Leu Met Asp Met Cys Ser Phe Asp Thr Ile Ser Thr Ser Thr Val
 245 250 255

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Asp Thr Lys Leu Ser Pro Phe Cys Asp Leu Phe Thr His Asp Glu Trp
 260 265 270

5 Ile Asn Tyr Asp Tyr Leu Gln Ser Leu Lys Lys Tyr Tyr Gly His Gly
 275 280 285

Ala Gly Asn Pro Leu Gly Pro Thr Gln Gly Val Gly Tyr Ala Asn Glu
 290 295 300

10 Leu Ile Ala Arg Leu Thr His Ser Pro Val His Asp Asp Thr Ser Ser
 305 310 315 320

Asn His Thr Leu Asp Ser Ser Pro Ala Thr Phe Pro Leu Asn Ser Thr
 325 330 335

15 Leu Tyr Ala Asp Phe Ser His Asp Asn Gly Ile Ile Ser Ile Leu Phe
 340 345 350

Ala Leu Gly Leu Tyr Asn Gly Thr Lys Pro Leu Ser Thr Thr Thr Val
 20 355 360 365

Glu Asn Ile Thr Gln Thr Asp Gly Phe Ser Ser Ala Trp Thr Val Pro
 370 375 380

25 Phe Ala Ser Arg Leu Tyr Val Glu Met Met Gln Cys Gln Ala Glu Gln
 385 390 395 400

Glu Pro Leu Val Arg Val Leu Val Asn Asp Arg Val Val Pro Leu His
 405 410 415

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Arg Gly Leu Ser Phe Ala Arg Ser Gly Gly Asp Trp Ala Glu Cys Phe
 35 435 440 445

Ala

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 <211> 96

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<212> DNA

<213> Daucus carota

<220>

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<222> (1)..(96)

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1 5 10 15

ttg ctt gta gta ttg gtg tca ctc aat ttg gct tcc gaa acc aca gct 96
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20 25 30

<210> 6

<211> 32

20 <212> PRT

<213> Daucus carota

<400> 6

Met Gly Arg Ile Ala Arg Gly Ser Lys Met Ser Ser Leu Ile Val Ser
25 1 5 10 15

Leu Leu Val Val Leu Val Ser Leu Asn Leu Ala Ser Glu Thr Thr Ala
20 25 30

30

<210> 7

<211> 93

<212> DNA

<213> Lupinus luteus

35

<220>

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<222> (1)..(93)

40 <400> 7

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Met Gly Tyr Tyr Ser Ile Tyr Cys Leu Ile Val Leu Val Asn Val Leu

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      1             5             10             15
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5             20             25             30

<210> 8
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10 <212> PRT
    <213> Lupinus luteus

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Val Phe Cys Asp Gly Gly Lys Thr Ser Ser Phe Val Arg Glu Ser
      20             25             30

20
<210> 9
<211> 1449
<212> DNA
<213> Artificial Sequence

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<220>
<223> Description of Artificial Sequence:chimeric
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<222> (1)..(1446)

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      1             5             10             15

ttg ctt gta gta ttg gtg tca ctc aat ttg gct tcc gaa acc aca gct      96
40 Leu Leu Val Val Leu Val Ser Leu Asn Leu Ala Ser Glu Thr Thr Ala
      20             25             30

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gcc atg ctg gca gtc ccc gcc tcg aga aat caa tcc act tgc gat acg   144
Ala Met Leu Ala Val Pro Ala Ser Arg Asn Gln Ser Thr Cys Asp Thr
      35              40              45

5  gtc gat cag ggg tat caa tgc ttc tcg gag act tcg cat ctt tgg ggc   192
Val Asp Gln Gly Tyr Gln Cys Phe Ser Glu Thr Ser His Leu Trp Gly
      50              55              60

caa tac gcg ccc ttc ttt tct ctg gca aac aaa tcg gcc atc tcc cct   240
10 Gln Tyr Ala Pro Phe Phe Ser Leu Ala Asn Lys Ser Ala Ile Ser Pro
      65              70              75              80

gat gtt cct gcc gga tgc cat gtc act ttc gcc cag gtt ctc tcc cgc   288
15 Asp Val Pro Ala Gly Cys His Val Thr Phe Ala Gln Val Leu Ser Arg
      85              90              95

cat gga gca cgg tat ccg acc gac tcc aag ggc aag aaa tac tcc gct   336
His Gly Ala Arg Tyr Pro Thr Asp Ser Lys Gly Lys Lys Tyr Ser Ala
      100              105              110

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Leu Ile Glu Glu Ile Gln Gln Asn Ala Thr Thr Phe Glu Gly Lys Tyr
      115              120              125

gcc ttc ctg aag aca tac aac tac agc ctg ggc gcg gat gat ctg act   432
25 Ala Phe Leu Lys Thr Tyr Asn Tyr Ser Leu Gly Ala Asp Asp Leu Thr
      130              135              140

ccc ttc gga gag cag gag ctg gtc aac tcc ggc gtc aag ttc tac cag   480
30 Pro Phe Gly Glu Gln Glu Leu Val Asn Ser Gly Val Lys Phe Tyr Gln
      145              150              155              160

cga tac gaa tcg ctc aca aga aac att gtc ccg ttc atc cga tcc tca   528
35 Arg Tyr Glu Ser Leu Thr Arg Asn Ile Val Pro Phe Ile Arg Ser Ser
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Gly Ser Asn Arg Val Ile Ala Ser Gly Asn Lys Phe Ile Glu Gly Phe
      180              185              190

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	Leu Asp Pro Gly Thr Cys Thr Val Phe Glu Asp Ser Glu Leu Ala Asp			
10	225	230	235	240
	gac atc gaa gcc aat ttc acc gcc acg ttc gtc ccc tcc att cgt caa			768
	Asp Ile Glu Ala Asn Phe Thr Ala Thr Phe Val Pro Ser Ile Arg Gln			
	245	250	255	
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	Arg Leu Glu Asn Asp Leu Ser Gly Val Ser Leu Thr Asp Thr Glu Val			
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	acc tac ctc atg gac atg tgc tcc ttc gac acc atc tcc acc agc acc			864
20	Thr Tyr Leu Met Asp Met Cys Ser Phe Asp Thr Ile Ser Thr Ser Thr			
	275	280	285	
	gtc gac acc aag ctg tcc ccc ttc tgt gac ctg ttc acc cat gaa gaa			912
	Val Asp Thr Lys Leu Ser Pro Phe Cys Asp Leu Phe Thr His Glu Glu			
25	290	295	300	
	tgg atc aac tac gac tac ctc cag tcc ccg aac aaa tac tac ggc cat			960
	Trp Ile Asn Tyr Asp Tyr Leu Gln Ser Pro Asn Lys Tyr Tyr Gly His			
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	ggc gca ggt aac ccg ctc ggc ccg acc cag ggc gtc ggc tac gct aac			1008
	Gly Ala Gly Asn Pro Leu Gly Pro Thr Gln Gly Val Gly Tyr Ala Asn			
	325	330	335	
35	gag ctc atc gcc cgt ctc acc cac tcg cct gtc cac gat gac acc agc			1056
	Glu Leu Ile Ala Arg Leu Thr His Ser Pro Val His Asp Asp Thr Ser			
	340	345	350	
	tcc aac cac aca ttg gac tcc aac ccg gct act ttc ccg ctc aac tcc			1104
40	Ser Asn His Thr Leu Asp Ser Asn Pro Ala Thr Phe Pro Leu Asn Ser			
	355	360	365	

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Pro Phe Ala Ser Arg Met Tyr Val Glu Met Met Gln Cys Gln Ser Glu
15                420                425                430

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Gln Glu Pro Leu Val Arg Val Leu Val Asn Asp Arg Val Val Pro Leu
    435                440                445

20  cat ggc tgt ccg gtt gat gct ttg gga aga tgt acg cgg gat agc ttc 1392
His Gly Cys Pro Val Asp Ala Leu Gly Arg Cys Thr Arg Asp Ser Phe
    450                455                460

25  gtg aag ggg ttg agc ttt gcc aga tct ggc ggt gat tgg gcg gag tgt 1440
Val Lys Gly Leu Ser Phe Ala Arg Ser Gly Gly Asp Trp Ala Glu Cys
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30  Phe Ala

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35  <212> PRT
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Leu Leu Val Val Leu Val Ser Leu Asn Leu Ala Ser Glu Thr Thr Ala

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	Gln Tyr Ala Pro Phe Phe Ser Leu Ala Asn Lys Ser Ala Ile Ser Pro		
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	Asp Val Pro Ala Gly Cys His Val Thr Phe Ala Gln Val Leu Ser Arg		
	85	90	95
15	His Gly Ala Arg Tyr Pro Thr Asp Ser Lys Gly Lys Lys Tyr Ser Ala		
	100	105	110
	Leu Ile Glu Glu Ile Gln Gln Asn Ala Thr Thr Phe Glu Gly Lys Tyr		
	115	120	125
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	130	135	140
	Pro Phe Gly Glu Gln Glu Leu Val Asn Ser Gly Val Lys Phe Tyr Gln		
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	Arg Tyr Glu Ser Leu Thr Arg Asn Ile Val Pro Phe Ile Arg Ser Ser		
	165	170	175
30	Gly Ser Asn Arg Val Ile Ala Ser Gly Asn Lys Phe Ile Glu Gly Phe		
	180	185	190
	Gln Ser Thr Lys Leu Lys Asp Pro Arg Ala Gln Pro Gly Gln Ser Ser		
	195	200	205
35	Pro Lys Ile Asp Val Val Ile Ser Glu Ala Ser Thr Ser Asn Asn Thr		
	210	215	220
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40	225	230	235
	Asp Ile Glu Ala Asn Phe Thr Ala Thr Phe Val Pro Ser Ile Arg Gln		
			240

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	260	265	270
5	Thr Tyr Leu Met Asp Met Cys Ser Phe Asp Thr Ile Ser Thr Ser Thr		
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	Glu Leu Ile Ala Arg Leu Thr His Ser Pro Val His Asp Asp Thr Ser		
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	Ser Asn His Thr Leu Asp Ser Asn Pro Ala Thr Phe Pro Leu Asn Ser		
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	Gln Glu Pro Leu Val Arg Val Leu Val Asn Asp Arg Val Val Pro Leu		
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	His Gly Cys Pro Val Asp Ala Leu Gly Arg Cys Thr Arg Asp Ser Phe		
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Phe Ala

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 20 25 30

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 35 40 45

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 50 55 60

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 Gln Tyr Ala Pro Phe Phe Ser Leu Ala Asn Glu Ser Val Ile Ser Pro
 65 70 75 80

gag gtg ccc gcc gga tgc aga gtc act ttc gct cag gtc ctc tcc cgt 288
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	85	90	95	
	cat gga gcg cgg tat ccg acc gac tcc aag ggc aag aaa tac tcc gct			336
	His Gly Ala Arg Tyr Pro Thr Asp Ser Lys Gly Lys Lys Tyr Ser Ala			
5	100	105	110	
	ctc att gag gag atc cag cag aac gcg acc acc ttt gac gga aaa tat			384
	Leu Ile Glu Glu Ile Gln Gln Asn Ala Thr Thr Phe Asp Gly Lys Tyr			
	115	120	125	
10	gcc ttc ctg aag aca tac aac tac agc ttg ggt gca gat gac ctg act			432
	Ala Phe Leu Lys Thr Tyr Asn Tyr Ser Leu Gly Ala Asp Asp Leu Thr			
	130	135	140	
15	ccc ttc gga gaa cag gag cta gtc aac tcc ggc atc aag ttc tac cag			480
	Pro Phe Gly Glu Gln Glu Leu Val Asn Ser Gly Ile Lys Phe Tyr Gln			
	145	150	155	160
	cgg tac gaa tcg ctc aca agg aac atc gtt cca ttc atc cga tcc tct			528
20	Arg Tyr Glu Ser Leu Thr Arg Asn Ile Val Pro Phe Ile Arg Ser Ser			
	165	170	175	
	ggc tcc agc cgc gtg atc gcc tcc ggc aag aaa ttc atc gag ggc ttc			576
	Gly Ser Ser Arg Val Ile Ala Ser Gly Lys Lys Phe Ile Glu Gly Phe			
25	180	185	190	
	cag agc acc aag ctg aag gat cct cgt gcc cag ccc ggc caa tcg tcg			624
	Gln Ser Thr Lys Leu Lys Asp Pro Arg Ala Gln Pro Gly Gln Ser Ser			
	195	200	205	
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	Pro Lys Ile Asp Val Val Ile Ser Glu Ala Ser Ser Ser Asn Asn Thr			
	210	215	220	
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	Val Asp Thr Lys Leu Ser Pro Phe Cys Asp Leu Phe Thr His Asp Glu	
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 450 455 460

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gtg agg ggg ttg agc ttt gct aga tct ggg ggt gat tgg gcg gag tgt 1440
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Ala Met Leu Ala Val Pro Ala Ser Arg Asn Gln Ser Ser Cys Asp Thr
 35 40 45

Val Asp Gln Gly Tyr Gln Cys Phe Ser Glu Thr Ser His Leu Trp Gly
 35 50 55 60

Gln Tyr Ala Pro Phe Phe Ser Leu Ala Asn Glu Ser Val Ile Ser Pro
 65 70 75 80

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 85 90 95

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Ala Phe Leu Lys Thr Tyr Asn Tyr Ser Leu Gly Ala Asp Asp Leu Thr
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 165 170 175

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 180 185 190

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 305 310 315 320

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5 Glu Leu Ile Ala Arg Leu Thr His Ser Pro Val His Asp Asp Thr Ser
 340 345 350

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Phe Ala Leu Gly Leu Tyr Asn Gly Thr Lys Pro Leu Ser Thr Thr Thr
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15 Val Glu Asn Ile Thr Gln Thr Asp Gly Phe Ser Ser Ala Trp Thr Val
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Pro Phe Ala Ser Arg Leu Tyr Val Glu Met Met Gln Cys Gln Ala Glu
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Gln Glu Pro Leu Val Arg Val Leu Val Asn Asp Arg Val Val Pro Leu
 435 440 445

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JOINT INVENTORS' DECLARATION FOR PATENT APPLICATION
AND POWER OF ATTORNEY

As the below named inventors, we hereby declare that:

Our residences, post office addresses and citizenship are as stated below our names.

We believe that we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled: *METHOD OF MODIFYING PLANT PRODUCTIVITY*.

the specification of which:

_____ is attached hereto;

X was filed on March 25, 2002 as Application Serial No. 10/089,364.

We hereby authorize our legal representative to add reference to the Serial No. and/or filing date of the above-referenced application to this declaration.

We hereby state that we have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

Prior Foreign Application(s)

We hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application(s) for patent or inventor's certificate having a filing date before that of the application to which priority is claimed:

Country	Application No.	Date of Filing (day,month,year)	Date of Issue (day,month,year)	Priority Claimed 35 U.S.C.119
Australia	PQ3049/99	24 September 1999		Yes <u>X</u> No <u> </u>

Prior Provisional Application(s)

We hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

Application Serial Number	Date of Filing (day,month,year)
------------------------------	------------------------------------

Prior U.S. Application(s) and PCT International Application(s) Designating the United States

We hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s), or § 365(c) of any PCT International application(s) designating the United States listed below:

Application Serial Number	Date of Filing (day,month,year)	Status (Patented, Pending, Abandoned)
PCT/AU00/01183	22 September 2000	Pending

Insofar as the subject matter of each of the claims in this application is not disclosed in the prior United States, foreign or PCT International application(s) to which priority has been claimed above in the manner provided by the first paragraph of Title 35, United States Code, §112, we acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.


We hereby appoint, both jointly and severally, as our attorneys and agents with full power of substitution and revocation, to prosecute this application and any corresponding application filed in the Patent Cooperation Treaty Receiving Office, and to transact all business in the Patent and Trademark Office connected herewith the following attorneys and agents, their registration numbers being listed after their names:

9 Lorance L. Greenlee, Reg. No. 27,894; Ellen P. Winner, Reg. No. 28,547; Sally A. Sullivan, Reg. No. 32,064; Donna M. Ferber, Reg. No. 33,878; G. William Van Cleave, Reg. No. 40,213; Susan K. Doughty, Reg. No. 43,595; Heeja Yoo-Warren, Reg. No. 45,495; Tamala R. Jonas, Reg. No. 47,688, and Mary Beth Vellequette, Reg. No. 47,903 all of Greenlee, Winner and Sullivan, P.C., 5370 Manhattan Circle, Suite 201, Boulder, CO 80303.

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States

Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

(1) Full Name of 1-00
First Inventor: Alan Edward RICHARDSON
Residence: Rivett, Australian Capital Territory
Citizenship: Australian AUX
Post Office Address: 3 Cassia Place
Rivett, Australian Capital Territory
AUSTRALIA

(1) Signature 

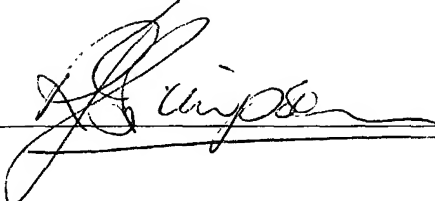
Date 5/June/2002

(2) Full Name of 2-0
Second Inventor: Julie Ellen HAYES
Residence: Euroa, Victoria AUX
Citizenship: Australian
Post Office Address: RMB 2730
Euroa, Victoria 3666
AUSTRALIA

(2) Signature 

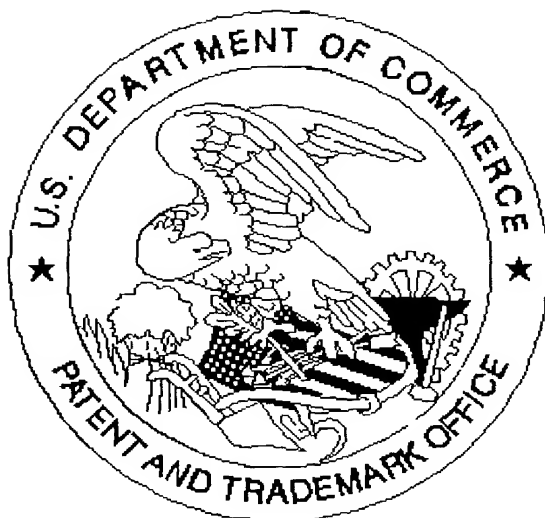
Date 21st June, 2002

(3) Full Name of 3-0
Third Inventor: Richard Joseph SIMPSON
Residence: Murrumbateman, New South Wales NSW AUX
Citizenship: Australian
Post Office Address: "Erinbila", Crisps Lane
Murrumbateman, New South Wales 2582
AUSTRALIA

(3) Signature 

Date 17/June/2002

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